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A Survey of the Present Status of Red Squill as a Rodenticide*,†

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By making use of a modified voluntary ingestion procedure, the dosage-mortality curves for both male and female rats were determined for 4 red squill standards of widely varying potency. The ratio of the LD_{50}'s for males to the LD_{50}'s for females was found to be a constant and the slopes of the male and female curves were in each case found to be parallel. It should, therefore, be possible for a bioassayist to determine this constant for his strain of rats, incorporate it in an adjusted dosage schedule and thereafter use both sexes in his squill assays.

ALTHOUGH the menace of disease and destruction caused by rodents has harassed the world for many centuries, it was not until the onset of the recent world conflict that a concerted and coordinated attack was made on the problem of rodent control. When the Mediterranean campaign of the Axis Powers cut off the United States from its normal supply of red squill, the hitherto staple rodenticide, an attempt to propagate a strain of squill bulbs in the Western Hemisphere was initiated. The approximate toxicity to rats of many hundreds of red squill bulbs was determined in this laboratory in order to select the most toxic ones for use in this project[1]. In the course of the screening program, several improvements have been made in the bioassay procedure. The purpose of this paper is to present a general review of the significant information available on red squill, an over-all evaluation of its usefulness as a rodenticide, the refined bioassay procedure including the data supporting the alterations made and the results obtained, and finally the case for the adoption of a standard of reference.

Red squill, *Urginea maritima* (L) Baker is an onion-shaped bulb that grows in the Mediterranean Basin. It is a uniformly dull, dark, purplish red perennial herb of the lily family with fibrous roots proceeding

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[1] These bulb samples were sent to this laboratory by the Wildlife Research Laboratory of the United States Department of Interior, Fish and Wildlife Service, Denver, Colo., where the samples were taken from a shipment of 1500 bulbs originally imported by the Board of Economic Warfare.

from the base of a large tunicated, pear-shaped bulb. The bulb is from 4 to 6 inches long, often weighing up to 4 lb. and grows half submerged in the sand. It is to be distinguished from a discolored white squill bulb by microscopic examination in which, in the case of red squill, cells with red coloring matter can be seen at irregular intervals (1).

Wild red squill bulbs have been gathered for many years by the natives of North Africa and sold for European use as a rodenticide. Its age-old popularity is due in a large measure to the fact that while it is poisonous to rodents it is relatively nontoxic to human beings and other forms of animal life. This property is due both to the violent emetic action of red squill on most animals except rodents, which are incapable of emesis, and the almost absolute refusal of domestic animals to voluntarily ingest toxic doses (2). Some investigators have published figures for the LD_{50}[2] of red squill on dogs, cats, and swine by stomach tube which would seem to discount the emetic activity as a safety factor (3, 4) but experiments conducted in this laboratory have demonstrated the existence and efficacy of both these safety factors in dogs. In an effort to obtain an estimate of the LD_{50} for dogs, females were given capsules containing amounts up to 10 times the LD_{50} for female rats. In all cases emesis was obtained in one to four hours, and the animals recovered after being only mildly ill. Other investigators have reported that dogs and other domestic animals will invariably refuse baits containing any sizable proportion of squill, even under conditions of extreme hunger (3–7). Although red squill has this important advantage as a rodenticide it has several drawbacks. One of the biggest of these is its relatively low toxicity to most species of rodents. The very best of the large number of squill bulb samples that have been received in this laboratory were found to have LD_{50}'s of the order of 350 to 500 mg./Kg. to male rats and 60 to 100 mg./Kg. for female rats. The unfavorable comparison with other rodenticides such as zinc phosphide with an LD_{50} of 47 mg./Kg., α-naphthylthiourea with an LD_{50} of 25 mg./Kg., thallium sulfate with an LD_{50} of 23 mg./Kg., and sodium fluoroacetate with an LD_{50} of 6 mg./Kg. is partially offset by the consideration that rats will voluntarily consume more squill than they will these other more toxic substances. It should also be remembered that from the point of view of long-range rodent control operations, it is much more important that the female population be controlled than the male. However, other things being equal, red squill will not kill off as high a percentage of the rodent population as these other more toxic rodenticides.

Another important disadvantage of red squill is the extremely wide variation in the toxicity of different bulb samples which necessitates their bioassay. Part of this variation between bulb samples is due to strain differences and part to the marked seasonal cycle in toxicity which red squill bulbs have been shown to undergo. Algerian bulbs, for instance, have been found to have a satisfactory toxicity to rats of less than 500 mg./Kg. while bulbs from other areas showed toxicities of 3000 mg./Kg. and up. On the other hand, even normally toxic Algerian samples were found to have little toxicity if they were harvested at the wrong time of the year. The optimal period for harvesting the bulbs is now known to be in the dry part of the summer when the leaves have died and the flowers are coming up—usually in August and September. If a toxic strain of red squill is selected, the bulbs harvested in the recommended period and then properly processed, the resulting powder is an efficacious rodenticide.

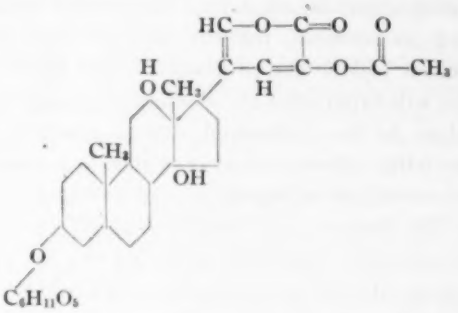
The commercial processing of red squill is relatively simple and inexpensive. As soon as possible after harvesting, the outer husks are removed and the bulbs sliced into transverse sections about $^1/_2$ to $^1/_4$ inch thick called "squames." The fresh squames are sometimes subjected to a treatment with the vapors of methyl alcohol and acetic acid which is supposed to destroy the enzymes believed to decompose the toxic principle during storage. Usually, however, the squames are simply dried in an oven at 80° to constant weight or more primitively

[2] LD_{50} is defined as the dose of the toxic substance per Kg. of body weight which will kill 50 per cent of a group of animals.

spread out in the sun to dry. This operation complete, the squill is ground to a powder that will pass a 40-mesh screen and is shipped in hermetically sealed containers (6). However, before a squill powder is used as a rodenticide its potency should be determined by biological assay.

In the event that the assay indicates the crude squill powder to be insufficiently toxic for use as a rodenticide, it is possible to take recourse in fortification. It was reported as far back as 1927 by Winton that the toxic principle of red squill is soluble in 80 per cent ethyl alcohol (8). This property is made use of in the fortification process in which the toxic principle is extracted using a countercurrent principle as described by Crabtree, Ward, and Garlough, and by Leblanc and Lee (9, 10). The extract is then concentrated by evaporation *in vacuo*, mixed with crude red squill, and the mixture dried at 80°. The fortified product has a very satisfactory toxicity and retains the important safety feature of the crude red squill. As a positive alternative the extract itself is sometimes marketed as a rodenticide for there is some evidence that this product has the additional advantage of not discouraging rats from returning to the bait in the event that they did not ingest a toxic dose at the first feeding (11).

Considerable work by various investigators has been devoted to an attempt at isolation and characterization of the toxic principle of red squill. The first attempts at isolation were reported in 1929 by Munch, Silver, and Horn who concentrated the toxic principle which they called "scillitin" by a process of extraction with alcohol, adsorption on charcoal, elution with 80 per cent alcohol and evaporation to dryness *in vacuo*. This substance was found to have an LD_{50} of 4 mg./Kg. for male rats which was better than 100 times the potency of their starting material (1). More advanced work in this direction was reported in 1942 by Stoll and Renz who succeeded in extracting, purifying, and isolating the principle which they named "scilliroside." They report that the pure principle is a bright yellow compound which is easily soluble in alcohols, glycols, dioxane, and glacial acetic acid; moderately soluble in acetone; and very slightly soluble in water, the hydrocarbons, chloroform, and ether. It decomposes at 168–170°. It was found to have an LD_{50} of 0.7 mg./Kg. when administered to adult male rats by stomach tube. Their efforts at characterization have been successful to the extent that they were able to report that scilliroside has a polyhydrocyclopentano phenanthrene nucleus attached to a lactone of 5 carbon atoms and 2 double bonds (12, 13). A possible structural formula according to their work is represented as follows:



The location of the twelfth oxygen atom and the double bonds is still in doubt, but apparently scilliroside is closely related to the cardioactive glucoside Scillaren A (12, 13).

Although the characterization of the active principle of red squill is nearly complete, little is known of its mechanism and site of action. However, the characteristic behavior of squill-poisoned rats is informative. Within two hours after ingestion of a surely fatal dose the animal becomes lethargic and will exhibit signs of irritation around the nose and mouth area. About two hours later his respiration will become labored, and approximately six hours after poisoning he will begin to gyrate. In this condition a rat will rotate violently on his longitudinal axis for brief intervals and then lie prostrate on the bottom of the cage after he has exhausted himself (3). A rat that has reached this stage will almost always die of respiratory failure in a short time (1), but occasionally rats demonstrate remarkable recuperative powers or sometimes continue to live for a week or more in a condition close to death. It is interesting to note that if the cage of a resting rat is struck, the rat immediately resumes gyrating (3). The organ-

ized character of the violent muscular activity indicates that the site of action of scilliroside is the central nervous system.

There is another phase to the problem of poisoning rodents besides toxicity which must be taken into account and that is making sure that rats ingest toxic doses. There are apparently two factors that determine this property of acceptance: one is the palatability of the substance at concentrations which it is desirable to use in baits; the other is the length of time after ingestion before a rat feels the first toxic effects. It is highly desirable that this time factor be as long as possible, for the shorter it is the greater will be the probability that the animal will experience these warning symptoms before he has consumed a toxic amount of the bait. Observation of numerous assays indicates that red squill is at least satisfactory in this respect, and from the standpoint of palatability rats have been known to eat voluntarily baits containing concentrations of squill up to 40 per cent (6).

Although adequate methods are not available for the accurate evaluation of many of the desirable characteristics of a rodenticide, the evidence available indicates that squill is important. As has been pointed out, its prime advantage is its specificity for rodents which allows it to be used in situations where other compounds would be too hazardous to human beings and domestic animals. Its cardinal disadvantages are the wide variation in toxicity between different batches of powders and the relatively low toxicity of even the more toxic or fortified samples. Again on the positive side are such desirable features as its satisfactory acceptability and its low cost of production. From the economic standpoint squill is perhaps the cheapest of the rodent poisons. In normal times squill bulbs cost 12 to 15 cents a pound and were imported at the rate of one-half million pounds yearly (14). Up until now, it has not been considered profitable to cultivate the plant because of the relatively small demand and the long period necessary for the bulbs to mature. As has been stated, there is a current effort at propagation of a suitable strain of squill in this hemisphere, but this attempt is not far enough along to determine whether it will result in a product of consistently high toxicity.

BIOASSAY

Because of the tremendous variation in the toxicity of squill bulbs and since the complexity of the squill toxic principle precludes the possibility of a chemical method for the determination of potency for the present, the necessity of the development of a method for biological assay of squill powders was recognized at an early date. In 1927 Winton first outlined his voluntary feeding bioassay method for squill (8) and with certain modifications this method is still used. This investigator also reported for the first time the difference in susceptibility that exists between male and female white rats. His observations that females are at least twice as susceptible as males to the action of the rodenticide has been confirmed by Crabtree, Ward, and Welsh whose work would indicate that the difference is due to the presence in the male rat of testosterone (15). They reported that castrate male rats were about as susceptible as female rats and that when these castrates were injected with testosterone they again became as resistant as normal males. Further work on this mechanism would be in order. There is no question that the sex difference actually exists and because of it most workers in the field have felt it necessary to restrict themselves to the use of male rats in their bioassay procedures (16, 17).

There has been some controversy among those who have published squill bioassay methods concerning the proper method for bait preparation. Munch, Silver, and Horn recommend feeding a weighed amount of squill mixed with ground diet equal to 1 per cent of the rat's body weight and maintain that in this way complete bait consumption is assured at all times (6); however, Ward, Crabtree, and Garlough (18) assert that diet and squill should be fed in constant ratio because at low dosage levels the excess amount of food will interfere with the toxicity of the very low percentage of squill in the bait. In this laboratory it was found that rats do not normally feed in relation to

their weight but that between the ages of one and three months eat approximately 15 Gm. of food per day. It was also found that when a constant percentage bait is fed, low doses necessitated such a small amount of bait that the error due to the amount which rats unavoidably waste in their feeding becomes appreciable. Therefore, it was decided that the most accurate as well as the simplest procedure would be to feed the appropriate amount of squill in a constant amount of diet. Four grams was determined to be the optimum for complete bait consumption and minimum waste.

Considerable work has been done on factors affecting the toxicity of red squill to rats which could enter in as errors in the bioassay method. Lubitz *et al.* (16) found no effect on toxicity with diets low in protein and high in carbohydrate, low in protein and high in fat, and baits with varying amounts of moisture. The toxicity is decreased by a diet high in protein and low in carbohydrate, and in baits with a high concentration of fat and baits containing pectin (16). Ward *et al.* have reported that altitude has a very considerable effect on toxicity of red squill to rats, but later qualified their findings to the specific strain of rats which they first used (19). It is important that all these factors be taken into account in the design of a red squill bioassay method.

EXPERIMENTAL

It was felt from the outset of the studies on red squill undertaken in this laboratory that the results obtained for the toxicity of a compound by voluntary ingestion were the most relevant to the study of rodenticides. For this reason, the method developed for use in the bioassay of squill samples and in the study of factors affecting the toxicity of red squill to rats to be later described represents a refinement and simplification of the procedure first published by Winton in 1927 (8).

The animals used for the bioassay were young healthy albino rats of a suitable 100 Gm.-weight range that had been held a minimum of two weeks in the laboratory under constant conditions and fed on Purina Dog Chow. They were segregated in individual cages and deprived of feed for a period of twenty-four hours but were given water. They were then weighed and their weights recorded for the purpose of computation of the doses.

If the squill sample to be assayed was in the form of dried squames, a powder was prepared by the use of a Wiley micro mill fitted with a 40-mesh screen. The individual doses were weighed out into the feeding jars. The jars found best suited for this purpose are 1-pint wide-mouth Presto Mason jars. Four grams of ground and screened Purina Dog Chow was then added to each sample and the bait mixed by allowing the jars to rotate on an inclined turntable for several minutes. These baits were fed the fasted rats in the late afternoon in order to approximate their normal feeding time. The following morning the empty jars were removed from the cages, the condition of each rat noted, and the surviving rats fed their normal daily ration. It has been found that with this procedure the fasted rats almost invariably consumed the entire bait. In the rare instances when this does not occur the animal is, of course, rejected. The animals were discarded after a six-day observation period.

During the course of the many assays in which this simple yet accurate procedure was used, it was found that red squill powders become hygroscopic when the relative humidity exceeds 70%. Under these conditions it was necessary to invert the order of the bait-mixing operations by immediately mixing the weighed amount of squill into the 4 Gm. of control diet which had been previously introduced into the feeding jars. In this way the squill powder was not given time to clump and a homogeneous bait was insured.

In order to secure the most information, the data obtained by this procedure were plotted in the form of dosage-mortality curves. In the use of this technique 10 animals at each of 3 to 5 dosage levels are normally sufficient for a suitable curve. The probit of the mortality ratio was plotted as the ordinate against the log dose as the abscissa and the best straight line was drawn among the points obtained (20, 21). From a curve such as this the LD_{50} of the sample or the dose that will kill any desired percentage of the animals may be read. This technique has the additional advantage of revealing the degree of animal variation as expressed by the slope of this straight line. From these curves it was concluded that the average slope of the dosage-mortality curve of red squill was sufficiently steep to permit the use of a screening procedure involving 3 rats (one each at 3 levels differing by a factor of 2, i.e., 500, 1000, and 2000 mg./Kg.), which in turn made it possible to select the 17 of the most toxic samples from a group of over 1300 different red squill bulbs.

RESULTS

One of the biggest disadvantages of previously published methods for the bioassay of red squill has been the restriction to the use of only male rats resulting in a great deal of waste in time and animals in laboratories extensively engaged in red squill work. Accordingly, a quantitative study was made of the difference in toxicity of red squill to male and to female white rats. Using the procedures and techniques outlined above, dosage-mortality curves were determined on both sexes for 4 squill standards.

TABLE I.—COMPARISON OF THE TOXICITY OF RED SQUILL TO MALE AND FEMALE RATS
(40 to 50 rats for each curve)

Standard No.	LD_{50} mg./Kg. Male	Female	Ratio M/F	Log Ratio	Slope = Probit / Log Dose Male	Female
2290	2818	423	6.66	0.82	6.3	6.9
2450	1728	309	5.59	0.75	5.6	5.4
214	525	87	6.03	0.78	8.6	8.5
621	524	89	5.89	0.77	7.0	6.3

The results obtained from these experiments are tabulated in Table I. From these results it may be seen that the ratio of the LD_{50} of male rats to the LD_{50} of female rats is, considering the wide range of potencies covered and the inherent error in the experiment, a constant for a single strain of rats and that the slopes of the curves are in each comparison similar. Further proof of the validity of this constant ratio is the fact that highly correlated results were obtained when 28 additional squill samples were assayed on both male and female rats by taking this constant into account in an adjusted dose schedule. With this dose schedule it was possible to screen more than 1300 squill samples using both sexes of rats. For any experimenter intending to do a large number of red squill assays it should therefore be possible to determine the ratio of the susceptibilities of the males to females in his strain of rats, and thereafter use both sexes in his bioassays.

For the purpose of providing a basis for selection of the optimum weight range for rats in the bioassay of red squill, a study of the comparative susceptibility of different sized rats was undertaken. Dosage-mortality curves were determined for 3 different weight ranges of male rats with the same squill standard, and the slopes and LD_{50}'s which were obtained are shown in Table II. These data reveal

TABLE II.—EFFECT OF SIZE OF RAT ON TOXICITY OF SQUILL

Male Rats, Wt. Range in Gm.	LD_{50}	Slope = Probit / Log Dose
100–150	1734	8.0
151–250	1728	5.6
251–350	1314	2.0

first that the large rats are more sensitive than medium-sized or small rats, and second that the slopes of the dosage-mortality curves decrease significantly as the rats become heavier (correlation coefficient, $r = -0.9998$; $p = 0.02$). (See Fig. 1.) The latter observation means that there is more individual variation in susceptibility to squill as the animals become more mature. Therefore, it was deemed advisable that young rats of a reasonably narrow weight range be used in the bioassay for optimum results. It is interesting to note that the rats in the 100 to 150 Gm.-range were at once the least susceptible and demonstrated the least degree of variation as evidenced by the larger value for the slope of the dosage-mortality curve. Since rats in this weight range have not normally reached the age of puberty, this would seem to be a contradiction of the theory that testosterone is responsible for the greater resistance of male rats to red squill.

Besides the previous unnecessary exclusion of female rats there remains another very serious fault in red squill bioassay procedure. At the present time this method measures the potency of a squill sample against the susceptibility of a given strain of rats. This is valid for comparative studies on the action of red squill conducted in one laboratory on one strain of rats, but experience has shown that there is very wide degree of variability among different strains of rats under different conditions. During the course of the bioassay work described in this paper, 2 squill samples which had been previously assayed in the Fish and Wildlife Research Laboratories at Denver, Colorado, were received here for assay. The values found for the LD_{50}'s of both samples at Denver and in this laboratory when assayed by similar voluntary ingestion procedures on male white rats are shown in Table III.

TABLE III.—COMPARISON OF RESULTS OF TWO LABORATORIES ON RED SQUILL BIOASSAYS

Sample No.	Division of Pharmacology, LD_{50}	Denver Wildlife Research Lab., LD_{50}
2290	2818	1500
2450	1718	600

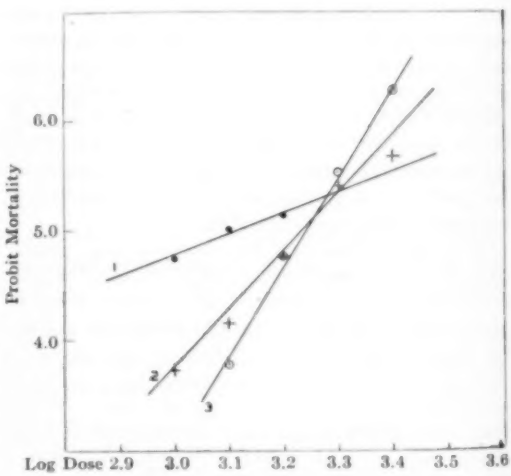


Fig. 1.—Key: 1 = Male rats 251–350 Gm.; 2 = Male rats 151–250 Gm.; 3 = Male rats 100–150 Gm.

Thus it is seen that the results of the two laboratories on the same squill samples differed markedly. A discrepancy such as this could only be resolved by the adoption of a satisfactory reference standard to which all samples of red squill could be compared. Such a procedure has also been urged by Munch, Ward, Mills, Buck, and Jarvis (17). There is some problem in this regard in that red squill is thought to undergo deterioration with age. However, it seems likely that under proper conditions of storage and handling, a standard could be used that would last for several years without losing its potency.

It is certain that the intercomparison of bioassay results from different laboratories will not be possible until a standard of reference for red squill is made available.

SUMMARY

1. A refined and simplified bioassay procedure for red squill on a voluntary feeding basis was described in detail.

2. It was found that the ratio of the LD_{50} of male to the LD_{50} of female white rats is a constant for a single strain of rats and that the slopes of the dosage-mortality curves are parallel. For these reasons the bioassay of red squill can be conducted with the use of both sexes of rats if this constant is determined and taken into account.

3. The toxic effects of red squill varied with the weight range of rats studied (100–350 Gm.), the greatest variation and lowest LD_{50} occurring with the largest rats. Therefore, rats for bioassay should be of a selected narrow weight range of young small rats.

4. The differences in the LD_{50}'s of the same sample as determined in two different laboratories are great. Before an intercomparison of bioassay results of different laboratories will be possible, a standard of reference must be made available.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Pinastric acid
Phloretin
3,3-Diethyl-2,4-pyridinedione
Thiolhistidine
o-Coumaric acid
3-Chloro-2-butanone
Adrenochrome
Phosphopyruvic acid
Phosphogluconic acid
Adenosine diphosphate
Fructose-6-phosphate
Phytadiene
Tetrahydrofarnesol
Tetrahydronerolidol
Neriin
Isoguanine
Promulsin
Hyaluronic acid
Homolevulinic acid
β-Chloroethyl vinyl ether
Phytyl alcohol and bromide
Carbobenzoxy chloride
Oxaldiureidoxime
l-Borneol

The Toxicity of Sodium 3,12-Dihydroxy-7-Cholanate and Its Influence on Bile Flow in Rats*

By WILLARD M. HOEHN and ROMAINE JOHNSON†

Keto-compounds obtained by oxidation of any hydroxyl group of cholic acid are less toxic than the parent compound. The toxicity of the sodium salt of 3,12-dihydroxy-7-ketocholanic acid is shown to be less than bile acid salts and to compare favorably with sodium dehydrocholate in hydrocholeretic properties.

RECENT chemical studies concerning the partial oxidation of cholic acid have led to the identification of 3,12-dihydroxy-7-ketocholanic acid (1–5). It has been shown that this acid is an intermediate in the oxidation of cholic acid by *Alcaligenes faecalis* isolated from the cecum of the guinea pig. This finding presents the possibility that the 7-keto-acid is a component of circulating bile of the guinea pig. No color test has been developed to date to demonstrate the presence of this new acid in bile and the ketone reagents generally used to indicate keto-groups have not given satisfactory results when applied to the pure acid. The presence of this acid has not been shown in any of the bile obtained from natural sources.[1]

EXPERIMENTAL

The oral toxicity of several unconjugated bile acids in the form of their sodium salts was determined. The acid to be tested was dissolved in dilute sodium hydroxide solution in amounts to produce a 10 to 20 per cent concentration. It was difficult to prepare such a concentrated solution of lithocholic acid because of its geling properties. The acids were administered daily, using the stomach tube (6), for a period of ten days. The animals used were Wistar strain white rats weighing between 110–30 Gm. each (7). Intravenous toxicities were determined with the same solutions and Table I presents these data together with those obtained recently by other investigators. Oxidation of hydroxyl groups attached to cholanic acid to form keto groups reduced the toxicity to a marked degree. It is noteworthy that oxidation of any one of the hydroxyl groups in cholic acid produces a less toxic compound. Conversion of the 7-hydroxyl to a ketone yields a product less toxic than dehydrocholic acid when administered intravenously as the sodium salt.

The availability of the new keto acid made possible the study of its effect on bile flow. Data are presented to demonstrate the influence of sodium 3,12-dihydroxy-7-ketocholanate on the bile flow in rats using the technique of Krayer (7). The solution of the sodium salt was injected intravenously. Several experiments were performed in which the salts of the bile were administered orally with unsatisfactory results which were probably due to low absorption of them.

According to the Krayer technique, bile collected for a two-hour period was compared with a second two-hour sample from the same animal. The first sample served as a control for the second. In addition, a group of uninjected rats was run to obtain control samples for the second two-hour period. The animal was maintained in a state of anaesthesia during the periods of bile collection.

At the end of the first period all animals except the controls were injected with the appropriate solutions of the sodium salts. Immediately following the injection, the second two-hour sample was collected.

The control groups of animals gave very satisfactory results for the purpose intended, as demonstrated in Table II.

Sodium 3,12-dihydroxy-7-ketocholanate (20% *w/v*) and sodium dehydrocholate (20% *w/v*) by the intravenous route caused an accelerated flow of bile within two to three minutes. With either compound the increase in flow reached a maximum when the rate of flow was at least three times the rate observed toward the end of the first two-hour period. After the peak was passed the rate of flow gradually decreased so that at the end of the two-hour period it was very nearly the same as the flow preceding the injection.

Accompanying the accelerated flow the color of the bile changed from a rich golden-yellow to a pale greenish yellow. The normal color of the bile returned before this flow was greatly reduced so that at the end of the two-hour period the color of the last of the second sample was of the same color as that collected during the first period. Data for bile collections concerned with the 7-keto-acid and dehydrocholic acid are found in Table II. The cholic acid in the residues was determined by the Pettenkofer procedure described by Schmidt (8, 9). A summary of the data concerned with the sodium

* Received April 17, 1946, from the Research Laboratories of George A. Breon & Co., Kansas City, Mo.

† We gratefully acknowledge suggestions made by Dr. Krayer of the Department of Pharmacology, Harvard University, for cannulating the bile duct of the rat. The study of toxicities of bile acids was initiated by Dr. C. W. Sondern and J. Leon Sealey in our laboratories and we appreciate having their data available for publication.

[1] Since this paper was submitted, it has been noted that Haslewood identified this acid in fresh cow bile (12).

TABLE I.—TOXICITIES OF UNCONJUGATED BILE ACIDS ADMINISTERED AS SODIUM SALTS

Cholanic Acid	Conc. of Soln. of Sodium Salt	Route of Administration: Intravenous M. L. D.$_{50}$ Mg./Kg.	Oral M. L. D.$_{50}$ Mg./Kg.	Intraperitoneal[a] M. L. D. Mg./Kg. Male	Intraperitoneal[a] Mg./Kg. Female	Intravenous[b] L. D.	Intravenous[b] Mg./Kg.
3-Hydroxy	1%	200 ..	400 ..	..	..	...	..
3-Hydroxy-12-keto	10%	360 ..	.. ..	..	..	...	..
3,12-Dihydroxy	1%	140 ..	1250 ..	100	160	15–20	..
3,12-Diketo	15%	215 ..	.. ..	440	480	150–180	..
3,6-Diketo[c]	10%	380 ..	.. ..	..	..	...	..
3,6-Dihydroxy	10%	425 ..	.. ..	..	..	...	..
3,7-Dihydroxy-chenodesoxycholic	15%	165 ..	.. ..	..	..	...	..
3,7-Diketo	5%	300 ..	.. ..	..	..	...	..
3,7,12-Trihydroxy	15%	300 ..	2500 ..	260	280	180–200	..
3,12-Dihydroxy-7-keto	10%	1800 ..	4500 ..	..	..	...	..
3,7-Dihydroxy-12-keto	5%	1500 ..	.. ..	..	..	...	..
3-Keto-7,12-Dihydroxy	5%	675 ..	.. ..	..	..	...	..
3-Hydroxy-7,12-Diketo	20%	1200 ..	.. ..	..	..	...	..
3,7,12-Triketo	20%	1150 ..	5000 [d]	2000	2000	...	..

[a] In mice (10).
[b] In rabbits (11).
[c] In alkaline the normal form is converted to the *allo*-form.
[d] Nearly all of material given found in stomach at autopsy.

TABLE II.—BILE FLOW IN RATS

No. of Animals	Compound: Mg./Kg.	Compound: Sodium Salt of	Weight of Bile (in Gm.): 1st 2-Hr. Sample	Weight of Bile (in Gm.): 2nd 2-Hr. Sample	Increase or Decrease	Portion of Toxic Dose	Per Cent Increase or Decrease of 2nd Sample Over 1st
8	..	.....	1.459	1.457	−0.002	...	−0.05
3	250	Dehydrocholic Acid	1.430	3.041	1.611	0.23	+113
3	380		1.546	3.809	2.263	0.35	+146
2	250	3,12-dihydroxy-7-ketocholanic acid	1.194	2.240	1.046	0.15	+87.5
6	380		1.479	3.445	1.966	0.23	+132
2	126	Hyodesoxycholic Acid	1.721	1.945	1.224	0.30	+10
3	126	Dehydrohyodesoxycholic Acid	1.715	0.204, −1.511	...	0.33	−88.5

salts of hyodesoxycholic and dehydrohyodesoxycholic acids is given to demonstrate a decrease in the bile flow by this technique.

It is noted that: (a) At 250 mg./Kg. and 330 mg./Kg. sodium dehydrocholate caused a greater flow of bile than the sodium 3,12-dehydroxy-7-ketocholanate given at the same weight doses. (b) Toxicity of acid appears to have some effect on the flow of bile. (At 23% of the toxic dose, sodium 3,12-dihydroxy-7-ketocholanate produced a slightly greater effect, than that caused by sodium dehydrocholate.)

SUMMARY

Oxidation of any hydroxyl group of cholic acid yields a keto-compound less toxic than the parent compound. The toxicity of the sodium salt of 3,12-dihydroxy-7-ketocholanic acid when compared with bile acid salts falls into the group of lower toxicity. The sodium 3,12-dihydroxy-7-ketocholanate compares favorably with sodium dehydrocholate in toxicity and hydrocholeretic properties.

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A Study to Determine the Toxicity of Fumaric Acid*,†

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Data are given on the effects of administering small amounts of fumaric acid to rats, guinea pigs, and human subjects daily for prolonged periods of time and to rats in larger doses for short periods. The results of the study described indicate a low degree of toxicity for fumaric acid and sodium fumarate. The possibility of using fumaric acid in place of tartaric acid in foods and drugs is suggested.

AMONG the many effects of the recent war was the cessation of the importation of crude potassium acid tartrate ordinarily obtained as a by-product of the wine-making industry. This material is the mother substance of tartaric acid which is widely used in industry, food technology, and medicine. Fumaric acid is one of the organic acids which has some promise of replacing tartaric acid, and is of particular interest not only because of its close structural chemical relation to tartaric acid but also because its production as a by-product in microbiologic chemical synthesis is largely independent of other industrial processes. Furthermore, fumaric acid is a normal constituent of tissues [Annau, *et al.* (1)]. Obviously, before introducing a new compound of this kind, to be employed as a substitute for as widely used a substance as is tartaric acid, adequate information regarding its possible toxicity must be obtained. In the present investigation data have been secured on the effects of small amounts of fumaric acid administered daily to rats, guinea pigs, and human subjects for prolonged periods and of larger doses administered to rats for short periods.

CHRONIC TOXICITY STUDIES

A. Rats

Young weanling rats weighing 40 to 50 Gm. each of the "Yale" strain and of a local albino strain were used for the chronic toxicity studies. All animals were kept in individual cages designed to prevent coprophagy and were allowed food and water *ad libitum*. At weekly intervals food consumption and body weight were determined. The ration consisted of a basal diet composed of purified food materials to which was added the desired supplement, fumaric acid, or sodium fumarate. The fumaric acid was added to give either a 0.1 or a 1.0% level and the sodium fumarate was added in an amount (1.38%) equimolar to the 1.0% fumaric acid supplement. The composition of the diets used is given in Table I.

TABLE I.—COMPOSITION OF DIETS[a]

	Control, Gm.	0.1% Fumaric Acid, Gm.	1.0% Fumaric Acid, Gm.	1.38% Sodium Fumarate, Gm.
Sucrose	100	100	100	100
Casein	200	200	200	200
Acetic acid	2	...	...	...
Fumaric acid	...	1	10	...
Sodium fumarate	...	...	...	13.8
Dextrose	398	399	390	386.2
Crisco	210	210	210	210
Salt mixture (Wesson)	40	40	40	40
Cod Liver oil (Mead's Standardized)	50	50	50	50

[a] In addition, each rat received 0.5 Gm. of brewers' yeast per day as the source of the B vitamins.

One series of rats was maintained on the diets for two years. At the end of the first six months one-half of the rats were autopsied and examined grossly and histologically for evidence of any toxic effect of fumaric acid. The remaining rats were continued on the experiment for the remainder of the two-year period. The second series of rats was maintained for a period of one year.

Hemoglobin determinations and enumeration of the red and white blood cells were made at intervals indicated in Table III. At autopsy careful gross examinations were made and sections of the liver, kidney, stomach, and spleen were prepared for microscopic examination. The ash content of the left femur was determined on the animals which had been on the fumaric acid diets for thirty-one weeks; the bone was removed at autopsy and, after as much of the adhering tissue as possible was dissected away, dried at 100°. The bone was then extracted with alcohol and with ether, weighed and ashed in a muffle furnace at 600°. The ash content was calculated on the dry, fat-free basis.

Results.—Rats of both strains which received the fumaric acid appeared to be healthy at the end of the

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first year. Toward the end of the second year the surviving animals became unkempt in appearance, with discolored fur which also became quite coarse. This was probably due to the advanced age of the animals since it appeared in all groups alike. Deaths which occurred in the various groups during the two-year period were due in practically all cases to respiratory infection. In the case of the local strain there were many deaths toward the end of the first year, which appeared to be due to a combination of diarrhea and terminal "pneumonia."

ously. The data (not included in Table II) demonstrated that neither fumaric acid nor sodium fumarate at the dose administered had a retarding effect on growth.

Blood Studies

At fourteen, thirty-one, and fifty-four weeks hemoglobin determinations were made on the blood of animals receiving fumaric acid. The results which are summarized in Table III show that neither fumaric acid when fed at either 0.1 or 1.0% levels

TABLE II.—GROWTH OF CONTROL AND FUMARIC ACID-TREATED MALE RATS

	Control			0.1% Fumaric Acid			1.0% Fumaric Acid		
Weeks on Diet	Number of Animals	Weight Av., Gm.	Range	Number of Animals	Weight Av., Gm.	Range	Number of Animals	Weight Av., Gm.	Range
1	14	52	(32–65)	14	54	(33–60)	14	51	(34–60)
4	14	161	(90–187)	14	150	(121–183)	14	164	(130–191)
8	14	323	(203–380)	14	306	(231–350)	14	320	(290–377)
12	14	422	(301–495)	14	396	(300–466)	14	405	(366–453)
16	12	425	(330–483)	12	421	(339–469)	12	424	(378–524)
20	14	433	(367–496)	13	397	(214–464)	14	439	(350–541)
24	14	473	(387–544)	13	439	(337–492)	14	475	(366–598)
28	14	484	(407–588)	14	455	(392–488)	14	494	(388–645)
32	14	504	(421–633)	14	470	(393–526)	14	516	(441–673)
36	7	501	(405–634)	7	495	(447–536)	6	568	(479–700)
40	6	511	(398–631)	7	507	(450–547)	7	554	(447–708)
44	6	510	(360–661)	7	516	(483–551)	7	582	(495–742)
48	6	518	(369–662)	7	536	(504–577)	7	588	(490–731)
52	4	554	(474–634)	6	536	(497–572)	6	576	(475–632)
60	4	520	(394–604)	5	504	(427–566)	5	582	(486–778)
68	4	540	(420–626)	4	535	(476–587)	5	624	(503–908)
76	4	531	(376–655)	3	541	(498–580)	5	613	(489–894)
84	3	553	(464–630)	3	516	(475–552)	4	625	(448–991)
92	2	510	(494–525)	2	504	(500–508)	3	474	(409–519)
100	..	...	.....	1	518	.....	2	382	(364–400)
106	..	...	.....	1	528	.....	2	395	(352–438)

TABLE III.—HEMOGLOBIN CONTENT OF BLOOD OF CONTROL AND FUMARIC ACID- OR SODIUM FUMARATE-FED RATS

	Control		0.1% Fumaric Acid		1.0% Fumaric Acid		1.38% Sodium Fumarate	
Weeks on Diet	Number of Rats	Hemoglobin, Gm. %	Number of Rats	Hemoglobin, Gm. %	Number of Rats	Hemoglobin, Gm. %	Number of Rats	Hemoglobin, Gm. %
14	20	15.7 (16.6–14.5)	20	15.6 (17.0–14.1)	20	15.7 (17.3–13.1)	..	....
31	10	15.0 (17.3–14.3)	10	14.6 (16.5–13.9)	10	14.7 (16.0–13.7)	14	16.2 (21.0–14.2)
54	8	15.2 (16.5–14.4)	11	14.8 (17.0–13.5)	10	14.8 (16.4–13.5)	5	15.7 (16.4–15.0)

Growth.—The animals which received diets containing either the 0.1% or 1.0% fumaric acid showed little if any change in rate of growth (see Table II) from the control rats which received 0.2% acetic acid. As the experiment approached the two-year period and many of the animals in all the groups died, there appeared to be some weight differences between the groups. However, when statistical methods are applied, it is found that even at the point of greatest divergence of the growth curves, the differences are not statistically significant. Inasmuch as a different strain of rats was used in the study of sodium fumarate, groups fed the control diet and the 1% acid diet were studied simultaneously.

nor sodium fumarate fed at the 1.3% level had any deleterious effect on hemoglobin formation. The results of the erythrocyte and leucocyte counts are given in Table IV. There is essentially no difference between the red or white blood cell counts of any of the groups.

Bone Ash.—Since fumaric acid might conceivably influence the absorption or retention of calcium, the bone ash of rats maintained on the various diets was determined. The results presented in Table V show that there is no difference in the amount of bone ash between any of the groups, and that all values are within the accepted range for adult rats in a normal state of nutrition.

TABLE IV.—ERYTHROCYTE AND LEUCOCYTE COUNTS ON CONTROL AND FUMARIC ACID FED RATS

Diet	Weeks on Diet	Number of Animals	Erythrocyte Count, M. per Cmm.	Leucocyte Count per Cm.
Control	23	14	8.775 (8.30–9.86)	13,204 (11,100–16,300)
0.1% Fumaric acid	23	14	8.915 (7.51–9.79)	13,877 (11,100–19,400)
1.0%- Fumaric acid	23	14	9.107 (7.48–10.87)	12,526 (5,475–18,275)

TABLE V.—BONE ASH OF CONTROL RATS AND OF RATS FED FUMARIC ACID

Group	Number of Animals	Ash Content of Dry Fat-Free Bone Gm. %	Range
Control	10	64.1	(61.6–68.4)
0.1% Fumaric acid	10	62.7	(61.5–65.6)
1.0% Fumaric acid	9	64.4	(56.4–75.4)

given twice a week, but the animals showed signs of ascorbic acid deficiency, with some deaths. At this time cabbage became available and was substituted for the lettuce. In addition, each animal was given orally, once a week, a solution containing 50 mg. of ascorbic acid.

Results.—Table VI represents a summary of the weights of the male control and test animals over a period of fifty-two weeks. There were too few female animals in each group to give a significant average value. The average growth values, though quite irregular, are more or less coincident. From these data it appears that fumaric acid in amounts consumed in this study exerts no detectable effect on the growth of guinea pigs.

A number of guinea pigs were bred in order to determine whether fumaric acid might have any effect on reproduction or lactation. Twelve young pigs were cast by the two females which received fumaric acid in the ration. The fathers were also fed the fumaric acid-containing diet. Seven of the second generation animals were males and 4 were females. The rate of increase in body weight of the males was

TABLE VI.—GROWTH OF CONTROL AND FUMARIC ACID-TREATED MALE GUINEA PIGS

	Control			1.0% Fumaric Acid		
Weeks on Diet	Number of Animals	Weight, Gm.	Range	Number of Animals	Weight, Gm.	Range
1	8	266	(236– 304)	19	275	(223– 333)
4	8	338	(278– 442)	20	359	(286– 426)
8	8	459	(308– 562)	20	472	(273– 596)
12	7	459	(290– 578)	15	512	(390– 620)
16	5	518	(400– 602)	11	518	(436– 636)
20	5	598	(454– 773)	11	591	(460– 692)
24	5	688	(554– 840)	10	656	(464– 774)
28	5	758	(610– 944)	9	754	(592– 860)
32	5	809	(600–1080)	9	791	(534– 932)
36	5	818	(550–1004)	9	809	(596– 932)
40	5	916	(656–1110)	9	873	(684–1030)
44	5	961	(760–1120)	9	903	(686–1116)
48	5	947	(720–1134)	8	917	(731–1108)
52	5	978	(776–1189)	8	970	(771–1187)

Other Studies.—At the end of thirty-one weeks on the experiment, one-half of the animals of the Yale strain were autopsied; no general gross abnormalities were noted. The molar teeth were examined grossly for erosion but none was found. The liver, kidney, stomach, and spleen were examined microscopically; no consistent abnormalities[1] were observed.

B. Guinea Pigs

Young guinea pigs weighing between 236 and 304 Gm. were maintained for fifty-two weeks on a diet consisting of ground rabbit chow (Purina). One per cent fumaric acid was added to the ration of the test group of animals. The guinea pigs were housed in groups of three in wire-bottom cages. During the first sixteen weeks of the experiment lettuce was considerably greater than that of the parent control group, probably because these animals received diets adequate in ascorbic acid during the entire growth period.

From the foregoing observation it is concluded that fumaric acid, fed at a 1% level, does not have any detectable toxic effect on growth, reproduction, or lactation in the guinea pig.

C. Human Subjects

The human subjects selected for this study were all cared for in a hospital for chronic diseases. Approximately half of them were ambulatory, and the remainder were confined to bed at least part of each day. None of the subjects selected had a disease primarily affecting the gastrointestinal tract and each was mentally capable of giving an accurate account of any unusual symptom which he might experience. The ages of the patients varied from twenty to ninety-one years of age, the majority being

[1] Appreciation is expressed to Drs. Arthur Amolsch and Mark Maun of the Department of Pathology for examining the tissues.

between the ages of 40 and 70. There were 42 females and 33 males. The entire group was given an unselected routine hospital diet. The daily dose of fumaric acid, 500 mg. as a compressed tablet, was administered by the nurse each day during the one-year period of observation. During this time, all of the patients were observed daily by a competent nursing staff and were interviewed at least twice each week by the physicians conducting the study. It may be emphasized that 56 of the 75 patients (74%) received fumaric acid daily for ten months or longer.

During the year period of study determinations of hemoglobin, erythrocyte, leucocyte, differential leucocyte, nonprotein-nitrogen, creatinine, and hepatic and renal function were made at three-month intervals. Urine studies were made at frequent intervals. Body weights were carefully followed.

Results: Blood Studies

Hemoglobin.—If the variation in hemoglobin was greater than or less than one gram of the original value before fumaric acid was administered, the change was interpreted as an increase or decrease, respectively. Otherwise no change was considered to have occurred.

An analysis of the changes in hemoglobin in the blood of these patients (Table VII) indicates that 41 of the 72 studies, or 54.6% of the total, showed no change. Nineteen patients, or 25%, showed improvement in the amount of hemoglobin in the blood, and 12 patients, or 16%, showed a drop in the hemoglobin. In three patients repeat studies of hemoglobin were not made. It is evident that there was no significant difference with respect to changes in the hemoglobin either in males or females. The greatest range of variation was a 4-Gm. increase and a 4-Gm. decrease.

Erythrocyte Count.—If the change in the number of red blood cells was greater than or less than 500,000 cells, it was classified as an increase or a decrease, respectively. Otherwise, it was considered that no change had occurred.

Red blood cells were studied in 69 of the 75 patients. Forty-six patients (67%) showed no change, 13 patients (19%) showed an increase in the number of red blood cells, and 10 patients (14%) showed a decrease in the number of red blood cells. In six patients repeat examinations of the blood counts were not made.

Leucocyte Count.—If the variation in the number of white blood cells was greater than or less than 2000 cells, it was classified as an increase or decrease, respectively. Otherwise it was recorded as no change.

The white blood cell count was repeated in 71 of the 75 patients who received fumaric acid. Of these, 43 patients (61%) showed no change in the white blood cell count, 18 patients (25%) showed an increase in the white cells, and 10 (14%) showed a decrease in the white cells. In 4 cases the number of white cells was not repeated.

Differential Leucocyte Count.—The only consistent change in the differential counts of these patients was an increase in the number of monocytes in 8 of the 71 cases studied; five of these were females and 3 were males.

Nonprotein-Nitrogen.—Nonprotein-nitrogen determinations were made in 71 of the 75 cases. In 65 of these (or 92% of the number studied) there was no change in the concentration of this factor in the blood, and in 6 patients (8% of the number studied) there was an increase in this value.

Creatinine—Creatinine blood values were determined in 71 of the 75 patients. In 69 of these (97%) there was no change in the concentration of this substance, and in 2 there was an increase in this value.

TABLE VII.—EFFECT OF FUMARIC ACID ON BLOOD OF HUMAN SUBJECTS

		No Change		Increase		Decrease		No Repeat	
		Number of Patients	Per Cent	Number of Patients	Per Cent	Number of Patients	Per Cent	Number of Patients	Per Cent
Hemoglobin	Males	16		9		7			
	Females	26		10		5			
	Total	42	54.6	19	25.4	12	16.0	3	4.0
Erythrocyte count	Males	18		6		5		4	
	Females	28		7		5		2	
	Total	46	61.4	13	17.3	10	13.3	6	8.0
Leucocyte count	Males	16		11		4		2	
	Females	27		7		6		2	
	Total	43	57.4	18	24.0	10	13.3	4	5.3

Urine Studies

In 11 of the 70 in which repeated urine examinations were made, a one plus albuminuria was found. Four of these were females and 7 were males. None of these patients showed any significant increase in the number of casts or in the number of red blood cells or leucocytes in the urinary sediment.

Renal Function

If the decrease was more than 15 below the normal range (60 to 75) it was interpreted as a decrease in this test of renal function. Otherwise it was classified as no change.

The phenolsulfonphthalein test of renal function was done in 70 of the 75 patients. In 57 of these, or in 81% of the number studied, there was no change, and in 13 cases (19% of the number studied) there was a decrease in this renal function test.

Hepatic Function

If the average of the two check determinations showed an increase or a decrease greater than 10% of the original value, it was interpreted as an increase or a decrease, respectively. Otherwise it was considered that no change in this function had occurred.

Of the 73 patients in whom repeated bromosulfalein tests of the liver function were done, one showed a significant decrease in the liver function.

It is evident from the foregoing data that the daily administration of 500 mg. fumaric acid for one year produced no detectable untoward effect in the group of 75 subjects studied.

ACUTE TOXICITY STUDIES

Procedure and Results

Adult hooded rats weighing 200–250 Gm. were used for this series of experiments. The animals received the test dose either orally by means of a stomach tube or intraperitoneally. If the animal survived a single dose, the test dose was given for three consecutive days and on the fourth day the animal was sacrificed and autopsied. Before autopsy a bromosulfthalein liver function test was carried out. The test dose of bromosulfthalein was 5 mg./Kg. and the procedure was modified to permit the use of smaller samples of blood. Small pieces of glass tubing which were sealed at one end were used as comparator tubes. Estimation of the dye retained was made in a comparator block with standard series of tubes. The method was checked by producing liver damage in rats by the administration of CCl_4 in olive oil. Any retention of less than 10% of the administered dye was considered normal. In no case was there any abnormal liver function found in the animals on this study.

Kidney function was estimated by obtaining blood urea values. The diacetyl monoxime method (Barker, 2) was used. In the rat the values obtained are high due to the presence of allantoin. However, this does not vitiate the comparative values of the different supplements on kidney function within the species.

On autopsy careful gross examination was made and sections of the liver, kidney, stomach, and spleen were taken for microscopic examination. Weights of the animal before and at the end of the four-day period were also obtained. During the test period the animals were allowed to eat a commercial dog food ("Friskies") ad libitum. In these studies tartaric acid was used as a reference substance for the comparison of toxicities.

Oral Administration

Fumaric and Tartaric Acid.—Groups of rats were given fumaric acid orally at dosages which were multiples of an arbitrarily selected 587 mg./Kg. "base" level. The acid was given as a concentrated suspension containing 587 mg./cc. At this concentration fumaric acid is quite insoluble and in order to get the amount in a volume small enough to administer orally to rats it was homogenized in water using a Waring Blendor. Some animals received as much as 7.05 Gm./Kg./day for three consecutive days without showing any toxic effects. This latter amount was so large that it had to be administered in a divided dose; 3.52 Gm. was the largest amount which could be administered easily in a single dose. The limiting factor in the testing of the toxicity of fumaric acid in the rat is the volume of the rat's stomach. The animals lost some weight on the highest dose of fumaric acid, but this may be due to the fact that the animals were administered large amounts twice a day, thus keeping the stomach full and giving the animals little opportunity to eat. The higher doses of fumaric acid also produced some laxation. Fumaric acid produced no gross or microscopic tissue abnormality when administered at any of the levels studied.

Tartaric acid was administered to animals at a dose level of 645 mg./Kg. and multiples of this dose. This amount is equimolar to the base dose of fumaric acid fed. Toxic symptoms appeared when the amount of tartaric acid fed was at the 1.29 Gm./Kg. level. Three of the five animals given this dose died after the second administration. Autopsy showed erosion of the gastric mucosa, the stomach being filled with blood; the intestine was also filled with a bloody fluid. When tartaric acid was fed three times the base level, which was 1.97 Gm./Kg., five out of the five animals tested died. All died after the administration of the second dose. Autopsies showed erosion of the gastric mucosa with the intestine and the stomach filled with blood. In most cases the kidneys were enlarged. Microscopic studies showed degeneration of the kidney in the proximal convoluted tubules. Also foci of degeneration were found on the surfaces of the stomach.

From these studies it would appear that oral fumaric acid is less than one-fourth or one-fifth as toxic as tartaric acid.

Sodium Fumarate and Tartrate.—The base level of sodium fumarate used was 808/mg./Kg., which was equimolar with 587 mg./Kg. of fumaric acid. Toxic symptoms were first noted at 8.08 Gm./Kg. or 10 times the base level. Three of the five animals given this dose level died. On autopsy these animals showed fiery-red lungs and considerable amounts of fluid in the gastrointestinal tract. Microscopic examination showed slight lymphocytic infiltration of the gastric mucosa. There was considerable laxation in some of the animals, their ab-

dominal surface being wet and soiled. In addition, animals given this amount of sodium fumarate lost weight during the three-day test period. If the same amount were given in a divided dose, no toxicity was noted. Sodium tartrate was given at equivalent doses. No toxic symptoms were noted except a moderate diarrhea, even at a dose level of 8.83 Gm./-Kg. No tissue changes were noted on autopsy. From these studies it appears that sodium fumarate may be somewhat more toxic than sodium tartrate when administered orally.

Intraperitoneal Administration

Fumaric acid was given intraperitoneally to 2 rats at a dose level of 587 mg./Kg. and both animals died within a hour after injection. Autopsy showed hemorrhagic spots on the intestine near the spot of injection. The surface of the liver appeared to be "seared." There was engorgement of the intestine and liver. No microscopic studies were made.

Intraperitoneal sodium fumarate produced death when given at a dose level of 2.42 Gm./Kg. Five of the five animals tested died within an hour after administration. The injection appeared to cause great pain and the animal went into a shock-like state soon after injection. The animal became cold and unsteady and in many cases appeared to lose consciousness. On autopsy, fluid was found in the abdominal cavity and a few small hemorrhages were observed on the intestine in the region of the injection. The body wall in this area was pitted with small hemorrhages. Microscopic examination showed passive congestion of the liver, kidney, lungs, and adrenal, but no general damage of the heart. If the dose was lowered to 1.62 Gm./Kg. the animals survived three consecutive injections and on autopsy showed no gross or microscopic injury. There were no changes in the level of blood urea nor in hepatic function.

Sodium tartrate when injected at a level which would be toxic in the case of sodium fumarate did not produce any toxic changes, although the animals did lose weight and had a pronounced diarrhea. The liver appeared lighter in color. Thus sodium fumarate appeared more toxic than sodium tartrate when given intraperitoneally.

DISCUSSION

The foregoing results emphasize the low toxicity of fumaric acid and its sodium salt. No apparent adverse effect resulted from the prolonged administration of small amounts of fumaric acid to either rats, guinea pigs, or human subjects. These results are in agreement with those obtained by Bodansky, Gold, and Zahm (3), and by Locke, Locke, Schlesinger, and Carr (4). The results of the "acute toxicity" studies are in agreement with this general thesis. It was found necessary to administer relatively enormous doses of fumaric acid or sodium fumarate before significant toxic manifestations were observed.

The present studies therefore indicate that fumaric acid or sodium fumarate could be safely used in place of the tartrates in food technology and in therapeutic preparations.

SUMMARY AND CONCLUSIONS

A study of the toxicity of fumaric acid and of sodium fumarate has been made employing the "chronic" and "acute" procedures.

Rats were fed free fumaric acid at a 0.1 or a 1.0 per cent level or sodium fumarate at a 1.38 per cent level in adequate synthetic diet for a one- to two-year period. No untoward effect was observed on growth, hemoglobin content of the blood, erythrocyte and leucocyte counts, bone ash, teeth, or microscopic appearance of the liver, kidney, stomach, and spleen.

Guinea pigs fed 1 per cent free fumaric acid for one year showed no variations in growth from controls. No impairment in reproduction or lactation was observed. No evidence of toxicity was found in second generation guinea pigs, also fed the 1 per cent fumaric acid diet from birth.

No evidence of toxic effects was observed in 75 carefully studied human subjects given 500 mg. fumaric acid daily for one year.

From these studies the conclusion is drawn that small amounts of free fumaric acid or sodium fumarate administered orally for prolonged periods have no detectable toxic effect in 2 species of animals or in man.

The "acute" toxic effects of large doses of fumaric acid and sodium fumarate have been investigated in the rat.

The oral toxicity of free fumaric acid is extremely low, 7.05 Gm. per kilo per day producing no direct effect. Tartaric acid is four or five times more toxic under comparable conditions. Sodium fumarate was somewhat more toxic orally than the free acid apparently because of severe catharsis and dehydration produced. The "LD_{50}" was approximately 8.0 Gm. per Kg. Death appeared to be due to a disturbed fluid balance and secondary pulmonary infection.

When administered intraperitoneally, free fumaric acid (587 mg. per Kg.) produced corrosion of the viscera and death, and sodium fumarate at a dose of 2.42 Gm. per Kg. produced "shock," changes in fluid distribution, and death. Sodium tartrate appeared less toxic than sodium fumarate when administered intraperitoneally.

These studies reaffirm the low toxicity of fumaric acid and of sodium fumarate.

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The Stability of Solutions of Urea Peroxide in Glycerol, Isopropanol, and Ethylene Glycol*

By ETHAN ALLAN BROWN, HAROLD A. ABRAMSON, MANUEL H. GORIN, HANS O. KAUFFMANN, and EDWARD S. SHANLEY

Data are presented which indicate that solutions of urea peroxide in glycerin, in ethylene glycol, and in isopropyl alcohol are stable under ordinary storage conditions for relatively long periods of time.

SINCE their discovery 150 years ago, many attempts have been made to stabilize peroxide compounds. Machu (1) lists almost 100 methods concerned with the addition of stabilizing substances to aqueous peroxide solutions in order to prevent their decomposition and yet not interfere with the use of peroxide solutions in true peroxidase systems. The decomposition of peroxide solutions is always due to the presence of so-called "catalysts," the rate being determined by the physical and chemical characteristics of these "catalysts" and of the temperature. The control of the rate of such decomposition has therefore been the subject of intense study. Studies of this type have been especially important not only in the subject of peroxide chemistry but also in the fields of wound antisepsis and bacteriology.

Since little is known of the stability of hydrogen peroxide in organic solvents, it occurred to us that with the use of urea peroxide as a solute (and a source of hydrogen peroxide), and substantially anhydrous glycerol and similar liquids as solvents, it might be possible, first, to obtain a liquid peroxide system of enhanced storage stability; and second, to control the rate of decomposition by catalase systems thus prolonging the action of hydrogen peroxide at the surface of the wounds. Although it is well known that urea peroxide deteriorates rapidly in the presence of water, and although glycerol has been added to *aqueous* hydrogen peroxide as a stabilizing agent (1), no studies have been reported on solutions of hydrogen peroxide in glycerol or the use of urea peroxide to prepare such solutions in solvents of high viscosity.

The present communication deals with preliminary observations of the stability of urea peroxide in glycerol, isopropanol, and ethylene glycol in the absence of substantial amounts of water. Subsequent papers, in press and in preparation, deal with other properties and especially with the antiseptic effects of solutions of this type.

EXPERIMENTAL

The first observation on the stability of such solutions was made when it was noted that a 10% solution of urea peroxide in glycerol, kept in a clear

* Received Oct. 1, 1945, from the laboratories of the Asthma Research Foundation, Inc., Boston, Mass., and the Buffalo Electro-Chemical Co., Inc., Buffalo, N. Y.

TABLE I.—TITRATION VALUES FOR UREA PEROXIDE IN GLYCEROL, ISOPROPANOL, ETHYLENE GLYCOL, AND PROPYLENE GLYCOL AT ROOM TEMPERATURE FOR PERIODS OF 1 TO 300 DAYS

Weight % Urea Peroxide, Gm.	Solvent	Wt. % Distilled Water	Start	50 Days	100 Days	150 Days	200 Days	250 Days	300 Days
8	C. p. glycerol	0	1.20	1.28	1.27	1.26	1.25	1.24	1.21
8	C. p. glycerol	0	1.35	1.35	1.34	1.32	1.30	1.26	1.23
8	C. p. glycerol	4.5	1.25	1.24	1.23	1.22	1.20	1.18	1.14
8	C. p. glycerol	9.0	1.14	1.14	1.13	1.12	1.11	1.10	1.08
8	C. p. isopropanol	0	0.83	0.80	0.78	0.75	..	..	..
8	C. p. ethylene glycol	0	1.30	1.30	1.29	1.28	1.16	..	..
4	Propylene glycol	0	0.71	0.71	0.70	0.69	0.67	..	..

TABLE II.—TITRATION VALUES FOR UREA PEROXIDE ALONE AND IN DISTILLED WATER AT 40° FOR PERIOD OF 1 TO 70 DAYS

	Weight % Active Oxygen							
	Start	10 Days	20 Days	30 Days	40 Days	50 Days	60 Days	70 Days
Urea peroxide (no solvent)	17.0	17.0	16.8	16.4	14.1	16.0	14.4	0
	Start	2 Days	7 Days	12 Days	15 Days			
Urea peroxide (10%) in distilled water	14.4	14.3	14.2	14.1	0			

glass-stoppered bottle at room temperature, in and out of sunlight, for two years, retained a considerable amount of its biological activity. It was decided to carry out laboratory studies on solutions of urea peroxide in glycerol, isopropanol, and ethylene glycol. A number of solutions were prepared and samples were stored in closed glass bottles at room temperature (in daylight but not in direct sunshine).

The active oxygen content of the samples was determined at intervals by an iodimetric method. Samples of approximately 1 Gm. were weighed into dry flasks, to which were then added 25 ml. of 25% H_2SO_4, 2 Gm. of potassium iodide and a drop of ammonium molybdate solution. The liberated iodine was then titrated with standard $N/10$ thiosulfate.

The data obtained in this fashion were plotted on coordinate paper. Values taken from the resulting curves are listed in Table I.

Crystalline urea peroxide exhibits some significant contrasts as compared with the solutions noted. The urea peroxide of commerce is a stable material which can be stored without loss for extended periods of time if it is kept dry and reasonably cool. The decomposition of crystalline urea peroxide is, however, strongly self-accelerating and, once it begins, goes to completion rapidly. Spontaneous decomposition will occur following prolonged storage at elevated temperatures and more quickly if the material becomes moist. The data listed in Table II is typical of such decomposition at 40°.

This behavior is in marked contrast to that of the various urea peroxide solutions. As shown above, the solutions decompose slowly and regularly. The glycerol solutions also tolerate some moisture content without marked effect upon their stability.

SUMMARY

It has been shown that solutions of urea peroxide in glycerol, ethylene glycol, and isopropanol are relatively stable under ordinary storage conditions. The stability of these solutions is superior in some respect to that of crystalline urea peroxide. The glycerol solution exhibits a particularly desirable combination of properties.

Concomitant studies in the field of bacteriology have shown this solution to possess bactericidal properties against both Gram-positive and Gram-negative bacteria (2, 3). Clinical studies have proved it useful in the treatment of chronic middle ear infections (4). Papers recently published are concerned with lack of irritant properties as shown by patch test techniques and by results achieved in infections of the skin (5).

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Amino Alcohols. XVII.* Arylethanolamines†

By ROBERT SIMONOFF‡ and WALTER H. HARTUNG

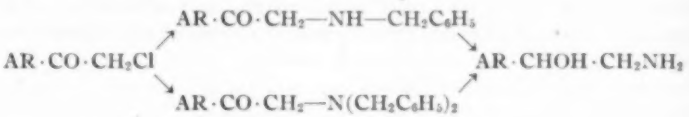
Aracyl chlorides, $Ar \cdot CO \cdot CH_2Cl$, were allowed to react with benzylamine or dibenzylamine to form aracylamines. These were then subjected to hydrogenolytic debenzylation, yielding primary aracylamines, $Ar—CO—CH_2—NH_2$; continued catalytic reduction, without previous isolation of the amino ketone, formed the corresponding arylethanolamines.

ARYLPROPANOLAMINES and arylethanolamines possess the optimum structure necessary for the production of pressor activity (1). The changes in pharmacodynamic activity produced in phenylpropanolamine by the introduction of various substituents into the phenyl nucleus have been extensively studied. In order to determine with greater certainty whether these modifications may be ascribed solely to the substituent under investigation, the effect of its presence in phenylethanolamine should also be investigated.

Many of the arylpropanolamines, $AR \cdot CHOH \cdot CH(CH_3) \cdot NH_2$, were prepared by the straightforward catalytic hydrogenation of the readily available oximinoketones of structure, $AR \cdot CO \cdot C(CH_3):NOH$ (2). Oximinoketones of structure, $AR \cdot CO \cdot CH:NOH$, may be converted with almost equal facility into the corresponding arylethanolamines, $AR \cdot CHOH \cdot CH_2NH_2$ (2, 3) but regrettably these desirable intermediates cannot be obtained by the methods so useful for the preparation of the higher homologs. Arylglyoxolohydroxamyl chlorides, $AR \cdot CO \cdot C(Cl):NOH$, are available (4), but as yet the problem of their consistent reduction to the desired amino alcohols is still unsolved.

In continuing the quest for a satisfactory procedure by which the arylethanolamines may be synthesized, the catalytic hydrogenolysis of benzylamines (5, 6) was studied. Aracyl halides, $AR \cdot CO \cdot CH_2X$, were condensed with benzylamine or dibenzylamine; the benzyl groups were removed catalytically, and the carbonyl group was reduced to the carbinol in the same reaction.

This method promises to be useful wherever it is possible to prepare the necessary aracyl halide.



EXPERIMENTAL

Preparation of Aracyl Chlorides

Phenacyl chloride was prepared by the Friedel-Crafts reaction, in a yield of 75%, by the action of chloroacetyl chloride on benzene.

p-Hydroxyphenacyl chloride was prepared by a Friedel-Crafts reaction on anisole according to the following method (3):

In a 2-liter, three-neck, round-bottom flask fitted with a reflux condenser connected to a HCl absorption trap, a sealed mechanical stirrer, and a small powder funnel were placed 60.5 cc. (0.55 mole) anisole, 48.2 cc. (0.63 mole) chloroacetyl chloride, and 1000 cc. ligroin (E.K. Co., practical). The reaction flask was immersed in a bath of water (40–45°) and during the course of forty-five minutes, 90.0 Gm. (0.675 mole) anhydrous aluminum chloride was added to the rapidly stirred mixture. After all of the $AlCl_3$ had been added, the mixture was heated in the water bath for an hour; stirring of the reactants and removal of the HCl being continued. The temperature of the bath was then raised to 50–55° and during the course of an hour a second portion of 90.0 Gm. anhydrous aluminum chloride was added. Then the bath was heated to boiling and the solvent recovered under reduced pressure (water-pump) during the course of one and one-half to two hours, the condenser remaining in the reflux position. When practically all of the ligroin had been removed, the reaction mixture was allowed to stand at room temperature for one hour and the aluminum complex decomposed by the addition of crushed ice followed by 500 cc. concentrated hydrochloric acid, during which, vigorous stirring of the reaction mix-

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ture was maintained. The dark purple, tarry mixture thus obtained was allowed to stand overnight and then extracted with six 300-cc. portions of ether. The ethereal extracts were combined, washed with 200 cc. of cold water, 250 cc. of a 5% solution of $(NH_4)_2CO_3$ and then with six portions (400 cc. each) of a 10% Na_2CO_3 solution to remove the *p*-hydroxyphenacyl chloride. The alkaline extracts were then treated individually with excess concentrated hydrochloric acid to precipitate the crude product. The precipitated product thus obtained was allowed to stand overnight in the refrigerator; it was then filtered off and dried with suction. Recrystallization from 80% ethanol with the addition of 3.0 Gm. of charcoal yielded 30.0 Gm. (31.6%) of *p*-hydroxyphenacyl chloride; m. p. 147–148° (dec.).

3,4-Dihydroxyphenacyl chloride was prepared according to the method of Levin (4).

Condensation of Aracyl Chlorides with Amines

ω-Benzylaminoacetophenone Hydrochloride.—In a 250-cc., glass-stoppered Erlenmeyer flask was placed 21.4 Gm. (0.2 mole) of benzylamine in 150 cc. of dry ether; then 15.0 Gm. (0.1 mole) of phenacyl chloride was added in small portions with shaking. After standing overnight the precipitated benzylamine hydrochloride was filtered off and washed with 25 cc. of dry ether. Absolute ethanolic hydrogen chloride was added to the filtrate to precipitate the amino ketone hydrochloride. The resulting precipitate was recrystallized from absolute ethanol and ether and 19.0 Gm. (72.75%) of white crystals, m. p. 215–219° (red effervescent melt), were obtained; this agreed with the reported melting point for ω-benzylaminoacetophenone hydrochloride (7).

ω-Dibenzylamino-*p*-hydroxyacetophenone Hydrochloride.—In a 500-cc., three-neck, round-bottom flask provided with a sealed mechanical stirrer, a reflux condenser, and a dropping funnel was placed 6.0 Gm. (0.04 mole) of *p*-hydroxyphenacyl chloride in 75 cc. of absolute ethanol. The stirrer was set in motion and the mixture warmed on a water bath until the *p*-hydroxyphenacyl chloride dissolved with the formation of a light tan color. Then 16.0 Gm. (0.08 mole) of dibenzylamine was added through the dropping funnel in 0.5-cc. portions. After the first addition of dibenzylamine the color changed to dark red and the mixture warmed up moderately, but not enough to cause the ethanol to reflux. The reaction mixture was then refluxed on a water bath for four hours, after which the mixture was allowed to stand overnight. The precipitated dibenzylamine hydrochloride was filtered off and the ethanol was completely removed under reduced pressure. The residue was taken up in 150 cc. of warm ether and the ethereal solution was washed with water to remove any trace of dibenzylamine hydrochloride. The ether was removed and an uncrystallizable oil was obtained; the oil was taken up in hot chloroform and filtered through a filter paper wet with chloroform to remove the water present. The chloroformic solution was cooled and absolute ethanolic hydrogen chloride added to precipitate the amino ketone hydrochloride. The resulting precipitate was recrystallized from absolute ethanol and ether and 11.5 Gm. (77.25%) of white crystals, m. p. 239–241° (dec.), were obtained.

Anal.—Calcd. for $C_{22}H_{22}O_2NCl$: N, 3.82%. Found (Kjeldahl): N, 3.79%; 3.81%.

ω-Dibenzylamino-3,4-dihydroxyacetophenone Hydrochloride.—In a 500-cc., three-neck, round-bottom flask provided with a sealed mechanical stirrer, a reflux condenser, and a dropping funnel was placed 18.6 Gm. (0.1 mole) of 3,4-dihydroxyphenacyl chloride in 100 cc. of absolute ethanol. The stirrer was set in motion and the mixture warmed on a water bath until the ketone had dissolved with the formation of a light purple color. Then 39.4 Gm. (0.2 mole) of dibenzylamine was added through the dropping funnel in 0.5-cc. portions. After the first addition of dibenzylamine the color changed to dark brown and the mixture warmed up moderately, but not enough to cause the ethanol to reflux. The reaction mixture was then refluxed on the water bath for six hours, after which the mixture was allowed to stand overnight. The precipitated dibenzylamine hydrochloride was filtered off and the ethanol was removed under reduced pressure. The residue was taken up in 200 cc. of warm ether and the ethereal solution was washed with water to remove any trace of dibenzylamine hydrochloride. The ether was removed and the crude product recrystallized from dilute ethanol. Thirty-two grams (92.25%) of white crystals, m. p. 165° (dec.), were obtained.

Anal.—Calcd. for $C_{22}H_{21}O_3N$: N, 4.03%. Found (Kjeldahl): N, 3.92%; 3.94%.

The free base was dissolved in absolute ethanol and absolute ethanolic hydrogen chloride was added to precipitate the ω-dibenzylamino-3,4-dihydroxyacetophenone hydrochloride, m. p. 203–205° (dec).

Attempts to prepare the monobenzylamino derivative were unsuccessful. Dakin (8) ascribed the failure of benzylamine and 3,4-dihydroxyphenacyl chloride to react as expected to the presence of the phenolic hydroxyl groups.

Catalytic Reduction of Benzylamino Ketones

Preparation of Catalyst.—The palladium-charcoal catalyst was prepared in sodium acetate solution by a modification of the general procedure employed in these Laboratories (9, 10).

ω-Aminoacetophenone Hydrochloride and Phenylethanolamine Hydrochloride.—The hydrogenation of ω-benzylaminoacetophenone hydrochloride in absolute ethanol stopped when the N-benzyl group was removed. When water was employed as a solvent, the benzyl group was removed and the carbonyl group was reduced to the secondary alcohol. These results are in harmony with previous observations that amino ketones are more easily reduced to amino alcohols in aqueous solution (11),

and would seem to indicate that hydrogenolysis of N-benzyl groups proceed more easily than reduction of the carbonyl group.

To 6.53 Gm. (0.025 mole) of ω-benzylaminoacetophenone hydrochloride, dissolved in 100 cc. of absolute ethanol, was added 3.0 Gm. of catalyst. The reduction mixture was shaken at room temperature under 100 lb. pressure for three hours when one mole of hydrogen had been absorbed. The catalyst was filtered off and the ethanol removed under reduced pressure. On recrystallization of the crude product from absolute ethanol and ether 3.75 Gm. (88.2%) of white crystals, m. p. 182° (dec.), were obtained. The product reduced Fehlings solution and formed an N-benzoyl derivative, m. p. 123–124° (12), thus indicating the product obtained was ω-aminoacetophenone hydrochloride.

These reduction conditions were repeated, except that distilled water was used instead of absolute ethanol. After one hour 2 moles of hydrogen had been absorbed. The product, isolated as described above, was recrystallized from absolute ethanol and ether, weighed 3.8 Gm., representing a yield of 87.7%, m. p. 211–212°; it formed an N-benzoyl derivative m. p. 145–146° (13), thus confirming the formation of phenylethanolamine hydrochloride.

p-Hydroxyphenylethanolamine Hydrochloride.—To 4.6 Gm. (0.0125 mole) of ω-dibenzylamino-*p*-hydroxyacetophenone hydrochloride, dissolved in 100 cc. of 95% ethanol, was added 3.0 Gm. of catalyst. The reduction mixture was shaken at room temperature under 150 lb. pressure for one hour when 3 moles of hydrogen had been absorbed. The catalyst was filtered off and the ethanol removed under reduced pressure. On recrystallization of the crude product from absolute ethanol and ether, 2.0 Gm. (85.8%) of greyish white crystals, decomposing at 168–170° in an effervescent melt, were obtained. The product formed a dibenzoyl derivative, m. p. 210° (14).

ω-Amino-3,4-dihydroxyacetophenone and 3,4-Dihydroxyphenylethanolamine Hydrochloride.—For the reduction of ω-dibenzylamino-3,4-dihydroxy acetophenone hydrochloride to the amino alcohol a higher ratio of catalyst was required. When the usual ratio of catalyst to ketone was employed, only the N-benzyl groups were removed.

To 4.0 Gm. (0.011 mole) of ω-dibenzylamino-3,4-dihydroxyacetophenone hydrochloride, suspended in 75 cc. of distilled water, was added 3.0 Gm. of catalyst. The reduction mixture was shaken at room temperature under 150 lb. pressure for one and one-half hours when 3 moles of hydrogen had been absorbed. The catalyst was filtered off and the solution evaporated to dryness over concentrated H_2SO_4, soda lime, and anhydrous $CaCl_2$ in an evacuated desiccator. An attempt was made to recrystallize the crude product from absolute ethanol and ether, but the material was too hygroscopic. The partially purified product was dried over P_2O_5 and 2.0 Gm. (88.5%) of tan crystals, m. p. 137° (dec.), were obtained. This evidence agrees with the melting point reported for 3,4-dihydroxyphenylethanolamine hydrochloride (15).

When the reduction was repeated using 9.5 Gm. (0.025 mole) of dibenzylamino ketone with the same amount of catalyst, only 2 moles of hydrogen were taken up in five hours. After removal of the catalyst the solution was concentrated over concentrated H_2SO_4, soda lime, and anhydrous $CaCl_2$ in an evacuated desiccator, to approximately one-tenth of the original volume. The concentrated solution was throughly chilled and 28% ammonia was added drop by drop until the solution was just neutral. The crude product was filtered off and washed throughly with cold water and ethanol. The product was dried over P_2O_5 and 3.5 Gm. (85.0%) of pinkish white crystals, m. p. 235° (dec.), were obtained. This evidence agrees with the melting point reported for ω-amino-3,4-dihydroxyacetophenone (16).

SUMMARY

The possibility of synthesizing arylethanolamines by hydrogenolytic debenzylation of suitable intermediates was examined. Aracyl chlorides were allowed to react with benzylamine or dibenzylamine to form, in good yields, compounds of general structure $AR \cdot CO \cdot CH_2—NH—CH_2C_6H_5$ or $AR \cdot CO \cdot CH_2—N(CH_2C_6H_5)_2$. The dibenzolamino derivative is more satisfactory for phenolic aracyl chlorides. Evidence indicates that with palladinized charcoal catalyst, the benzyl groups are more easily removed than is the carbonyl group reduced to carbinol.

The following intermediate benzylamino ketones were prepared and described:

1. ω-benzylaminoacetophenone hydrochloride.
2. ω-dibenzylamino-*p*-hydroxyacetophenone hydrochloride.
3. ω-dibenzylamino-3,4-dihydroxyacetophenone hydrochloride and converted into the corresponding arylethanolamines, *viz.*:
4. Phenylethanolamine hydrochloride.
5. *p*-Hydroxyphenylethaolamine hydrochloride.
6. 3,4-Dihydroxyphenylethanolamine hydrochloride.

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Pyrethrum Culture Investigations in Nebraska*,†

By ARTHUR E. SCHWARTING‡

The results of an investigation with Pyrethrum concerned with spacing, yield, and potency of plots of this plant under experimental cultivation in the several altitude ranges of the state are presented.

THE RENEWED interest in the growing of pyrethrum in the western hemisphere and the possible adaptability of the plant as a crop to the several altitude ranges and irrigated areas of the state prompted the present study. In normal times the requirements of pyrethrum are largely obtained from Dalmatia, Japan, Kenya, and Brazil. Commercial exploitation of the plant as a crop in the United States is limited, although considerable cultivation is in progress of development in Central and South American countries.

The present paper presents the results of a limited investigation of plantings of one seed source of pyrethrum (*Chrysanthemum cinerariaefolium* Vis.) obtained from the Division of Drug and Related Plants of the United States Department of Agriculture. The original work was inaugurated in 1942. Seedlings obtained from flats sown in the first week in February were transplanted into the field in early June in the several years of plantings. Replicated plots were established at the Scottsbluff Field Station in a spacing and yield study under irrigation. There were four replicated five-row plots of each of a six-inch, twelve-inch, eighteen-inch and twenty-four-inch spacing in rows eighteen feet long, the rows thirty inches apart. The field was uniform with respect to fertility and physical conditions. All other data were obtained from nonreplicated plots.

The harvesting of the flowers was done by hand. The middle row of the replicated plots was used to furnish yield data and the flowers collected were used for the assay determinations. Plants selected at random from the nonreplicated plots furnished the material used to assay these plots. These results (Table I) and other data are presented. The assay procedure (1) was conducted within three months of the harvest date and the average of three determinations is recorded.

The acre yields are calculated, based on full stands. Actual stands in the plots were lower. There was little winter killing, the

* Received July 15, 1946, from the University of Nebraska College of Pharmacy, Lincoln, Nebr.
† These investigations have been cooperative with the University of Nebraska Chemurgy Project.
‡ Instructor, Department of Pharmacognosy, College of pharmacy, The University of Nebraska.

TABLE I.—COMPARISON OF PLANTINGS

Sample	Location	Elevation (Feet)	Treatment	Age of Plants (Years)	Number of Flowers per Plant (Average)	Yield per Acre (Pounds)
1	Lincoln	1148	Nonreplicated plot	1	63	...
2	Lincoln	1148	Nonreplicated plot	2	74	...
3	North Platte	2800	Nonreplicated plot	1	..	...
4	North Platte	2800	Nonreplicated plot	2	49	...
5	North Platte	2800	Nonreplicated plot	3	64	...
6	Alliance	3971	Nonreplicated plot	1	..	...
7	Scottsbluff	3800	Nonreplicated plot	1	..	...
8	Scottsbluff	3800	Nonreplicated plot	2	..	...
9	Scottsbluff	3800	Replicated plots. Irrigated. Plants 6 in. in the row	1	48[a]	572[a]
10	Scottsbluff	3800	Replicated plots. Irrigated. Plants 12 in. in the row	1	69[a]	328[a]
11	Scottsbluff	3800	Replicated plots. Irrigated. Plants 18 in. in the row	1	74[a]	295[a]
12	Scottsbluff	3800	Replicated plots. Irrigated. Plants 24 in. in the row	1	75[a]	216[a]

[a] Data shown are calculated from average totals of 4 replications.

TABLE II.—PYRETHRIN CONTENT OF EXPERIMENTAL PLOTS

Sample	Crop	Pyrethrins (Moisture-free Basis)			
		Pyrethrin I	Pyrethrin II	Total	Ratio, I:II
1	1945	0.53	0.58	1.11	1:1.09
2	1946	0.55	0.58	1.13	1:1.05
3	1943	0.44	0.34	0.78	1:0.77
4	1944	0.43	0.59	1.02	1:1.37
5	1945	0.42	0.58	1.00	1:1.38
6	1943	0.66	0.44	1.10	1:0.66
7	1943	0.44	0.41	0.85	1:0.93
8	1944	0.34	0.52	0.87	1:1.56
9	1945	0.56	0.38	0.94	1:0.69
10	1945	0.57	0.39	0.95	1:0.68
11	1945	0.55	0.39	0.95	1:0.71
12	1945	0.54	0.34	0.88	1:0.63

largest loss of plants occurring just prior to flowering. Severe hail damaged the Scottsbluff plantings in the bud stage and this damage possibly contributed much to the death of many of the plants.

The data presented in Table II illustrate the assay results. Considerable variation is shown in the ratio of pyrethrin I and pyrethrin II. The altitude ranges of the plantings show little difference in the production of total pyrethrins.

The plantings in general proved to be quite unsuccessful and the project was discontinued. Of the several spacing studies incorporated in the project the twelve-inch spacing under irrigation appeared to be the best. There was smaller loss of plants and the hedge-like growth appeared to prevent desiccation during dormancy and between irrigations.

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An Assay for Choline Chloride in Pharmaceutical Products*

By WALTER C. GAKENHEIMER and ROSE M. REGUERA

A simple and accurate gravimetric procedure for the assay of choline chloride, as such, in various pharmaceutical products, has been developed. The method involves the precipitation of choline phosphotungstate from an anhydrous alcohol solution. The application of the assay procedure to aqueous solutions, elixirs, syrups, tablets, and capsules containing choline chloride is described and results are tabulated which indicate an accuracy of ±4%.

BECAUSE of the recent interest in the oral administration of choline for the treatment of cirrhosis of the liver (1, 2) and infective hepatitis (3), the need for suitable dosage forms of choline chloride has developed. Pharmaceutical development of a therapeutic agent requires an accurate method of studying the stability of the substance when combined with various carriers. There are many published assays designed for the determination of choline in plant or animal matter (4–7) or in pharmaceuticals (8), but these methods are not sufficiently accurate and are unnecessarily complicated for a stability study of choline chloride in various types of pharmaceuticals.

Drummond (9) reported the use of choline phosphotungstate in studying the separation of complex mixtures of bases and amino acids, and we have found this salt most suitable for our purpose. Choline phosphotungstate may be precipitated and filtered from absolute alcohol, dried, and weighed as $(C_5H_{15}O_2N)_3 \cdot H_3PO_4 \cdot (WO_3)_{12}$, the composition of which has been confirmed by microanalyses:[1]

	Theory for $(C_5H_{15}O_2N)_3 \cdot H_3PO_4 \cdot (WO_3)_{12}$	Found
Carbon	5.55%	5.59%
Hydrogen	1.49%	1.52%
Nitrogen	1.30%	1.41%
Phosphorus	0.96%	0.90%
Tungsten	68.01%	68.05%

This precipitate is slightly soluble in aqueous or hydroalcoholic solutions, but sufficiently insoluble in absolute alcohol for the development of this analytical method. Consequently, care is exercised to remove moisture from the assay sample.

Since 1 mole of choline phosphotungstate (mol. wt. 3244.57) is equivalent to 3 moles of choline chloride (mol. wt. 139.57), the theoretical factor for converting the weight of choline phosphotungstate to choline chloride is: $3 \times 139.57/3244.57 = 0.1291$. On the basis of the analyses of several samples of known choline chloride content, we have found that the use of the theoretical factor gave low results. This we have attributed to the slight solubility of choline phosphotungstate in absolute alcohol. Empirically, by using solutions representing 0.01 Gm. of choline chloride per cc., we have found that the conversion factor should be increased from 0.1291 to 0.1375 to compensate for the slight solubility of choline phosphotungstate under the assay conditions described below.

The only special reagent required for this assay is a filtered 10 per cent solution of phosphotungstic acid (C. P.) in absolute alcohol. Although pure anhydrous ethyl alcohol was used in this work, denatured anhydrous ethyl alcohol has been found to be satisfactory. Since, on a molecular basis, many phosphotungstic acids are theoretically possible (10), samples of this acid from eight different production lots manufactured during the past three years were treated with known quantities of choline chloride; the weights of the precipitates varied less than ±0.7 per cent.

It is necessary that a blank determination be made to assure the absence of interfering substances in the pharmaceutical product being analyzed. In testing several common elixirs, syrups, aromatic waters, and tablet and capsule fillers, we have encountered no interfering substances.

* Received July 9, 1946, from the Research Laboratories, Merck & Co., Inc., Rahway, N. J.

[1] The authors wish to express their appreciation to Mr. Richard N. Boos for these microanalyses.

EXPERIMENTAL

Assay Procedures

1. For liquids, such as solutions in water, syrups, elixirs, or aromatic waters, an accurately measured sample containing approximately 1.00 Gm. of choline chloride is placed in a 100-cc. volumetric flask. The sample is then heated on a steam bath for fifteen minutes under reduced pressure (100–125 mm.) to drive off moisture and interfering decomposition products, such as trimethylamine, if present. The residue is shaken with 50 cc. of absolute alcohol and, on cooling, sufficient of this solvent is then added to make 100 cc. After thorough mixing, 5 cc. of the clear supernatant liquid is added to 10 cc. of the phosphotungstic acid solution. The resulting precipitate is digested on a steam bath for five minutes, transferred to a fritted-glass Pyrex filter crucible (fine porosity), and washed with 5 cc. of absolute alcohol. After drying at 55° for two hours, it is weighed.

$$\text{Per cent } (w/v) \text{ choline chloride} = \frac{\text{wt. precipitate} \times 0.1375 \times 20}{\text{cc. vol. of sample}} \times 100$$

In the case of liquids containing a high percentage of ingredients which are insoluble in alcohol, it is advisable to use a graduated Pyrex cylinder in place of a volumetric flask so that, after dilution with absolute alcohol, the insoluble material may be thoroughly pulverized with a stirring rod.

2. For solids, such as tablets and capsules, an accurately weighed sample of the powdered material, containing approximately 0.50 Gm. of choline chloride, is transferred to a micro Soxhlet apparatus and extracted with 20 cc. of absolute alcohol for three hours. The extract is transferred quantitatively to a 50-cc. volumetric flask, carefully concentrated by distilling off the alcohol, and heated under reduced pressure (100–125 mm.) at 100° for fifteen minutes.

TABLE I.—ASSAYS OF CHOLINE CHLORIDE IN VARIOUS TYPES OF PHARMACEUTICALS

Type of Preparation	Theoretical Content	Assay
Capsule	0.300Gm./Cap.	0.305Gm./Cap.
		0.306 "
Tablet	0.250Gm./Tab.	0.256Gm./Tab.
		0.261 "
		0.262 "
Solution (Distilled Water)	0.050Gm./cc.	0.049Gm./cc.
		0.049 "
		0.049 "
Solution (Peppermint Water)	0.083Gm./cc.	0.085 "
		0.085 "
		0.086 "
Elixir	0.250Gm./cc.	0.238 "
		0.242 "
		0.242 "
Syrup	0.250Gm./cc.	0.241 "
		0.241 "

The residue is redissolved in sufficient absolute alcohol to make 50 cc. From this point, the procedure is identical with that described for the assay of liquid preparations, with the variation in calculation:

$$\text{Per cent } (w/w) \text{ choline chloride} = \frac{\text{wt. precipitate} \times 0.1375 \times 10}{\text{wt. of sample}} \times 100$$

The result is readily converted to the choline chloride content per tablet or capsule.

DISCUSSION

This assay procedure has been applied to several pharmaceutical preparations containing choline chloride. The results, as recorded in Table I, indicate that choline chloride can be determined within an accuracy of ±4% by this method.

The results obtained by this procedure in an assay of choline chloride in Peppermint Water were compared with results obtained by the U. S. P. XII method of assay (8). They are recorded in Table II and indicate good agreement between the two methods.

TABLE II.—COMPARISON OF PHOSPHOTUNGSTIC ACID METHOD AND U. S. P. XII METHOD FOR ASSAY OF CHOLINE CHLORIDE IN PEPPERMINT WATER

	Gm/Cc.
Theoretical Content	0.083
Assay by Phosphotungstic Acid Method	0.085
	0.085
	0.086
Assay by U. S. P. XII Method	0.083

SUMMARY

A gravimetric method for the assay of choline chloride in several pharmaceutical preparations is described. The method is comparatively simple, accurate, and does not require any special apparatus or reagent other than phosphotungstic acid.

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The Extraction of Caffeine from Pharmaceutical Preparations*

By KENNETH E. HOLT

Advantage is taken of the relatively low solubility of caffeine in ether to separate it from other drugs with which it is frequently combined in tablets and capsules. The method was developed previously by the author for caffeine, aspirin, and acetophenetidin in simple admixture and is applied here to mixtures containing in addition to caffeine one or more of the following: ephedrine, acetanilid, camphor, monobromated camphor, phenobarbital, and aspirin. The principle may be extended to preparations containing other alkaloids as well.

CAFFEINE is one of the more common ingredients found in pharmaceutical preparations usually in combination with such compounds as acetylsalicylic acid, acetanilid, phenobarbital, camphor, etc. Because of the similarity in physical properties the separation of caffeine from these compounds is often a laborious and time-consuming procedure.

In a recent article (1, 2) a method of separation of caffeine, acetylsalicylic acid, and acetophenetidin was proposed which decreased the time of separation of these compounds by several hours over that required by the A. O. A. C. method (3) without loss in accuracy. The proposed method utilizes the relative solubility of caffeine in ether, chloroform, and water. The approximate solubility of caffeine in the above solvents in terms of cc. of solvent per gram of caffeine is: chloroform 6 cc., water 50 cc., and ether 550 cc. (4). This gives a ratio of approximately 1 to 10 between the water-ether phase and also the water-chloroform phase. The solubility of caffeine in water is increased by acids and decreased by alkalies, thereby increasing the above ratios.

The method used for the separation of caffeine from acetylsalicylic acid and acetophenetidin has been successfully applied to other pharmaceuticals containing caffeine. The results of some of these experiments are given in the accompanying tables. The procedure used in these experiments is essentially the same as previously reported (1, 2) with minor changes in sample sizes and volumes of solvents depending upon the solubility ratios of the ingredients present. Because of the small amount of caffeine usually found in pharmaceuticals, the minute quantities of caffeine retained by the ether extracts will result in a high percentage error if the size of sample and volumes of solvents used are not held rather closely to those used in the following procedures.

The ingredients used in these experiments were desiccated U. S. P. grade chemicals and were mixed in the approximate proportions usually found in preparations of this type. Purified (fat extraction) ether and U. S. P. grade chloroform were used, both were redistilled before using and the ether was washed with water to remove any alcohol present.

EXPERIMENTAL

Separation of Caffeine, Acetanilid, and Ephedrine

Acetanilid.—Accurately weigh a sample containing 0.3–0.5 Gm. of acetanilid and transfer to a separatory funnel. Add 25 cc. of HCl (1 + 10) and extract with purified ether using one 40-cc. portion and five 20-cc. portions. Combine ether extracts in another separatory funnel and wash with one 10-cc. portion HCl (1 + 10) and one 10-cc. portion of water, washing these in turn through two 25-cc. portions of ether and adding to the original acid solution. (Save the acid solution for caffeine and ephedrine.) Filter all ether extracts through a cotton pledget into a tared flask. Evaporate ether, dry at 100°, cool, and weigh as acetanilid.

Caffeine.—Extract the original acid solution with four 30-cc. portions of chloroform, washing each in turn through 10 cc. water. Filter the chloroform extracts through a cotton pledget into a tared flask. Evaporate the chloroform, dry at 100°, cool, and weigh as anhydrous caffeine.

Ephedrine.—Transfer the original acid solution containing the ephedrine to a 300-cc. Kjeldahl flask, dilute to about 100 cc., and add 15 cc. 50%

* Received May 31, 1946, from the Chemical Laboratories, Cole Chemical Company, St. Louis 8, Mo.

NaOH. Steam distill the ephedrine collecting in an excess of standard acid solution according to the method by Schoen (5).

The results of experiments with caffeine and acetanilid and with caffeine, acetanilid, and ephedrine are given in Table I.

Filter all ether extracts through a cotton pledget into a tared flask. Evaporate ether, dry at 100°, cool, and weigh as phenobarbital.

Caffeine.—Extract the original acid solution with four 30-cc. portions of chloroform, washing each in turn through 5 cc. 2% NaOH and 5 cc. water.

TABLE I.—SEPARATION OF CAFFEINE, ACETANILID AND EPHEDRINE MIXTURES

Caffeine			Acetanilid			Ephedrine HCl		
Gm. Present	Gm. Found	%	Gm. Present	Gm. Found	%	Gm. Present	Gm. Found	%
0.1007	0.1008	100.1	0.6077	0.6069	99.9	...	...	...
0.0478	0.0488	102.1	0.5216	0.5220	100.1	...	...	...
0.0304	0.0305	100.3	0.5231	0.5238	100.1	...	...	...
0.0540	0.0528	97.8	0.5105	0.5077	99.5	0.2005	0.1990	99.2
0.0371	0.0376	101.3	0.2600	0.2636	101.4	0.2227	0.2214	99.4

Many preparations containing acetanilid and caffeine also contain camphor or monobromocamphor which would be extracted by the ether along with the acetanilid. These may be removed first by boiling the acid solution of the sample gently until the odor of camphor is absent, cooling and extracting as described above.

Any alkaloid which gives an acid salt, such as morphine, codeine, etc., may be substituted for the ephedrine in the above preparation. These will be retained in the acid solution and are readily extracted after the removal of the caffeine by rendering the solution alkaline.

Filter the chloroform extracts through a cotton pledget into a tared flask. Evaporate the chloroform, dry at 100°, cool, and weigh as anhydrous caffeine.

Acetylsalicylic Acid.—Dilute the $NaHCO_3$ solution to 100 cc., add 20 cc. of 5% NaOH, and boil fifteen minutes. Cool and dilute in a volumetric flask. Transfer an aliquot containing about 65 mg. acetylsalicylic acid to an iodine flask and proceed using the bromate method (6).

The results of experiments with caffeine and phenobarbital and with caffeine, phenobarbital, and acetylsalicylic acid are given in Table II.

TABLE II.—SEPARATION OF CAFFEINE, PHENOBARBITAL, AND ACETYLSALICYLIC ACID MIXTURES

Caffeine			Phenobarbital			Acetylsalicylic Acid		
Gm. Present	Gm. Found	%	Gm. Present	Gm. Found	%	Gm. Present	Gm. Found	%
0.0235	0.0229	97.5	0.0541	0.0544	100.6	...	...	...
0.0288	0.0284	98.6	0.0592	0.0604	102.0	...	...	...
0.0234	0.0229	97.9	0.1014	0.1039	102.4	...	...	...
0.0280	0.0278	99.3	0.1018	0.1032	101.4	...	...	...
0.0356	0.0351	98.6	0.1050	0.1062	101.1	...	...	...
0.0491	0.0485	98.8	0.1120	0.1129	100.8	0.4974	0.5022	101.0
0.0238	0.0234	98.3	0.0579	0.0596	102.9	0.4895	0.4860	99.3
0.0552	0.0538	97.5	0.0448	0.0439	98.0	0.2015	0.2012	99.9

Separation of Caffeine, Phenobarbital, and Acetylsalicylic Acid

Phenobarbital.—Accurately weigh a sample containing 0.05 Gm. to 0.10 Gm. of phenobarbital and transfer to a separatory funnel. Add 25 cc. of HCl (1 + 10) and extract with purified ether using four 25-cc. portions. Combine ether extracts in another separatory funnel and wash with one 10-cc. portion HCl (1 + 10) and one 10-cc. portion of water, washing these in turn through one 25-cc. portion of ether and adding to the original acid solution. (Save the acid solution for caffeine.) Combine the ether solutions and extract the acetylsalicylic acid with one 25-cc. and one 15-cc. portion of 5% $NaHCO_3$ and one 10-cc. portion of water, washing each through two 25-cc. portions of ether and combining in a 300-cc. Erlenmeyer flask. (Save the $NaHCO_3$ solution for acetylsalicylic acid.)

Acetylsalicylic acid when present in a pharmaceutical usually represents the major portion of the preparation and traces of it will remain in the acid solution after extraction with ether. These traces, while negligible in the acetylsalicylic acid analysis, will introduce a large percentage error in the caffeine analysis unless removed from the chloroform extracts by the 2% NaOH wash solution.

The solubility ratio of phenobarbital in water-ether is considerably greater than acetophenetidin or acetanilid; therefore, the smaller volume of ether used above will completely extract the phenobarbital, while the amount of caffeine extracted will be reduced to a fraction of a milligram.

Ephedrine or any acid-soluble alkaloid added to the above combination will be retained in the original acid solution and can be analyzed as indicated previously.

SUMMARY

A previously published method on the separation of acetylsalicylic acid, acetophenetidin, and caffeine has been adapted to other pharmaceuticals in which caffeine is an ingredient.

The pharmaceutical mixture is dissolved in an acid solution and ingredients such as acetanilid, phenobarbital, and acetylsalicylic acid are extracted with ether. The caffeine is then removed from the acid solution with chloroform, the acid solution retaining any ephedrine or other alkaloids present.

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The Identification of an Acid in the Root Bark of *Viburnum Prunifolium**

By JAMES M. HOLBERT

The major portion of the acid from *Viburnum prunifolium* root bark has been identified as isovaleric acid. The identification was made by the method of mixed melting points in which the anilide of the acid from *Viburnum prunifolium* root bark was compared to the anilides of known acids. The melting points of the anilides of the valeric and caproic acids are given.

IN A PARTIAL investigation of the contents of the root bark of *Viburnum prunifolium* Heyl and Barkenbus (1) reported the isolation of a valeric acid as the major portion of the total acid content. Since the identification of the acid was based on the analysis of its silver salt and since other work (2) suggested a caproic rather than a valeric acid, it was decided to reinvestigate the problem in order definitely to classify the acid as to kind and isomeric form.

The obvious approach to the problem was to convert the unknown acid to a derivative which in turn could be compared by the method of mixed melting point to the same derivative of known acids. Since only valeric and caproic acids were to be considered, the problem was resolved into the following steps: first, obtaining all isomers of valeric and caproic acid; second, converting these acids to a suitable derivative; third, isolating the major acid portion from the root bark of *Viburnum prunifolium* and subsequently converting it to the same derivative as the known acids; and fourth, identifying the unknown derivative by the method of mixed melting point.

EXPERIMENTAL

Preparation of the Acids.—All of the valeric acids, *n*-valeric, isovaleric, methylethylacetic, and trimethylacetic, and three of the caproic acids, *n*-caproic, isocaproic, and diethylacetic, are available from Eastman. The remaining five caproic acids were prepared by the following methods.

Methyl-*n*-propylacetic Acid.—This acid was prepared by the oxidation of 2-methylpentanol-1 with boiling 50% nitric acid.

sec-Butylacetic Acid.—This acid was prepared in fair yields by means of the malonic ester synthesis from *sec*-butyl alcohol (3).

Dimethylethylacetic Acid.—The preparation of this acid was accomplished by means of the Grignard synthesis according to the method of Putambeker and Zoellner (4, 5).

* Received June 3, 1946, from the Organic Research Laboratory, Chattanooga Medicine Company, Chattanooga, Tenn.

ter-Butylacetic Acid.—This acid was prepared in excellent yields from a sample of methyl-neopentyl ketone furnished the author by Dr. Frank C. Whitmore of Pennsylvania State College. The ketone is readily converted to *ter*-butylacetic acid and bromoform by the haloform reaction using sodium hydroxide and bromine (6).

Methylisopropylacetic Acid.—The preparation of this acid involved the dialkylation of malonic ester with isopropyl bromide and methyl bromide followed by saponification and decarboxylation.

Preparation of the Derivatives.—The anilides were chosen as the most suitable derivative for the above acids since they were all solids of convenient melting point. The anilides were prepared from the acids according to directions given in Shriner and Fuson (7). Melting points of the anilides as determined by the capillary tube method are as follows:

Anilide of Acid	M. p., °C. (uncor.)
n-Valeric	62.5–63
Isovaleric	109.5–110.5
Methylethylacetic	110–110.5
Trimethylacetic	129–130
n-Caproic	96–96.5
Isocaproic	111–112
Diethylacetic	124.3–125
Methyl-*n*-propylacetic	94–95
sec-Butylacetic	86–86.5
Dimethylethylacetic	89–90
ter-Butylacetic	130–130.3
Methylisopropylacetic	95–96

These melting points check closely with the values given in the literature except in the case of the anilide of methylisopropylacetic acid, which is given as 87.4° (8, 9).

Isolation of an Acid from Root Bark of Viburnum prunifolium.—The starting material was a basic, aqueous concentrate from the extraction of 30 lb. of *Viburnum prunifolium* root bark. To 2.4 kg. of the concentrate, representing 10 to 12 lb. of bark, was added 500 cc. of 20% sulfuric acid. This mixture was refluxed for ten hours and then steam distilled. Approximately 1.5 L. of distillate was collected. The distillate was made basic and concentrated to 50 cc. This material was acidified and repeatedly extracted with ether. The combined ether extract was dried over magnesium sulfate. The ether was removed by evaporation and the residue fractionated through a small column. The bulk of the material (0.6 Gm.) boiled at 87° at 28 mm. This material was converted to the anilide (7) and recrystallized several times from hot alcohol-water mixture. The anilide melted at 109.5–110.3°.

Identification of the Acid Isolated from Viburnum prunifolium Root Bark.—By comparing the melting point of the unknown anilide with the melting points of the known anilides, it is obvious that only three possibilities need be considered, namely, the anilides of isovaleric, m. p. 109.5–110.5°; methylethylacetic, m. p. 110–110.5°; and isocaproic, m. p. 111–112°. Small samples of these anilides were intimately mixed with an equal amount of the unknown anilide. Capillary melting points of these mixtures were taken with the following result:

Mixture	M. p., °C.
Isovaleranilide — unknown anilide	109.5–110.5
Methylethylacetanilide — unknown anilide	86–87
Isocaproanilide—unknown anilide	91–96

Since isovaleranilide is the only anilide that did not show a melting-point depression when mixed with the unknown anilide, the two must be identical and the acid obtained from *Viburnum prunifolium* root bark is identified as isovaleric acid.

SUMMARY

The major portion of the acid of *Viburnum prunifolium* root bark has been identified as isovaleric acid. The melting points of the anilides of the valeric and caproic acids are given.

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The Effect of Agar upon the Germicidal Efficiency of the Quaternary Ammonium Salts*

By ROBERT A. QUISNO, IRVIN W. GIBBY, and MILTON J. FOTER

To determine the effect of agar upon the germicidal potency of quaternary ammonium salts, 0.2 per cent agar was included in the germicide-bacteria mixtures during the test period. Standard germicide tests without agar were included as controls. When the four quaternary ammonium salts were tested in the presence of 0.2 per cent agar, germicidal dilutions were much lower than germicidal dilutions in the absence of agar. The neutralization effect of agar probably accounts for this discrepancy. Standard tests for antiseptic potency call for evaluation of creams, ointments, powders, etc., by agar cup plate methods. It is evident that such procedures are inappropriate for preparations which contain quaternary ammonium germicides.

DURING an investigation of death rates of bacteria treated with quaternary ammonium salts, it was observed that results obtained with liquid media and results obtained on agar plates were not in agreement. It therefore appeared advisable to determine the effect of agar upon the germicidal potency of these compounds.

EXPERIMENTAL

Dissolved agar and undissolved granular agar were added separately to the germicide dilutions prior to the addition of the test organism. Standard germicide tests performed according to the method outlined in Circular No. 198 of the U. S. Dept. of Agriculture were conducted for comparison. The determinations were made at 37°. A standard resistance strain of *Staphylococcus aureus* was used as the test organism. Results shown in the tables are averages of three tests in duplicate.

TABLE I.—EFFECT OF AN AGAR SOLUTION AND UNDISSOLVED GRANULAR AGAR UPON GERMICIDAL DILUTIONS OF QUATERNARY AMMONIUM SALTS

	Germicidal Dilution, No Agar	Germicidal Dilution, 0.2% Agar Solution	Germicidal Dilution, 0.2% Undissolved granular Agar
Zephiran[a]	1:38,000	1:8000	1:11,500
Phemerol[b]	1:32,000	1:10,000	1:8000
Cetamium[c]	1:80,000	1:15,000	1:11,500
Ceepryn[d]	1:85,000	1:14,000	1:10,000

[a] High molecular alkyl-dimethyl-benzyl-ammonium chloride.
[b] *para* - Tertiary - octyl - phenoxy - ethoxyethyl - dimethyl benzyl-ammonium chloride monohydrate.
[c] Cetyl trimethyl ammonium bromide.
[d] Cetyl pyridinium chloride.

The results shown in Table I demonstrate that the addition of agar to the germicide mixtures caused a sharp reduction in the germicidal activity of the four quaternary ammonium salts tested. The germicidal dilution of Zephiran was 1:38,000 without agar and 1:8000 in the presence of 0.2% agar solution. Germicidal dilutions of Phemerol, Cetamium,[1] and Ceepryn[2] were reduced from 1:32,000, 1:80,000, and 1:85,000 to 1:10,000, 1:15,000, and 1:14,000, respectively, by 0.2% agar solution. It is of interest to note that undissolved granular agar reduced the germicidal activity of the compounds tested to about the same degree as did the agar solution.

TABLE II.—BACTERICIDAL EFFICIENCY OF FILTRATES FROM MIXTURES OF GRANULAR AGAR AND GERMICIDE DILUTIONS

	Germicidal Dilutions		
Compound	Control, No Agar	0.2% Granular Agar, Filtered	0.2% Granular Agar, Unfiltered
Ceepryn	1:65,000	1:16,000	1:18,000

TABLE III.—CONCENTRATIONS OF CEEPRYN IN FILTRATES FROM AGAR-GERMICIDE MIXTURES

Preparation	Chemical Analysis
0.2% Agar + 0.02% Ceepryn	0.003% Ceepryn
0.2% Agar + 0.01% Ceepryn	0.003% Ceepryn
0.2% Agar + 0.005% Ceepryn	0.002% Ceepryn
0.2% Agar + 0.0033% Ceepryn	0.0016% Ceepryn
No Agar 0.01% Ceepryn	0.01% Ceepryn
No Agar 0.003% Ceepryn	0.003% Ceepryn

The effect of various concentrations of agar upon the germicidal potency of Ceepryn was investigated. Dissolved agar was added to germicide mixtures to give final concentrations of 0.2, 0.5, and 1.5%. During the test period the latter concentration was held at 47° while all other combinations were held at 37°. Germicidal dilutions of Ceepryn were, re-

* Received July 1, 1946, from the Department of Bacteriology, Research Laboratories of The Wm. S. Merrell Company, Cincinnati, Ohio.

[1] Cetyl trimethyl ammonium bromide is manufactured and sold under the registered trade name "Cetamium" by J. T. Baker Chemical Company.
[2] Cetyl pyridinium chloride is manufactured and sold under the registered trade name "Ceepryn" by The Wm. S. Merrell Company.

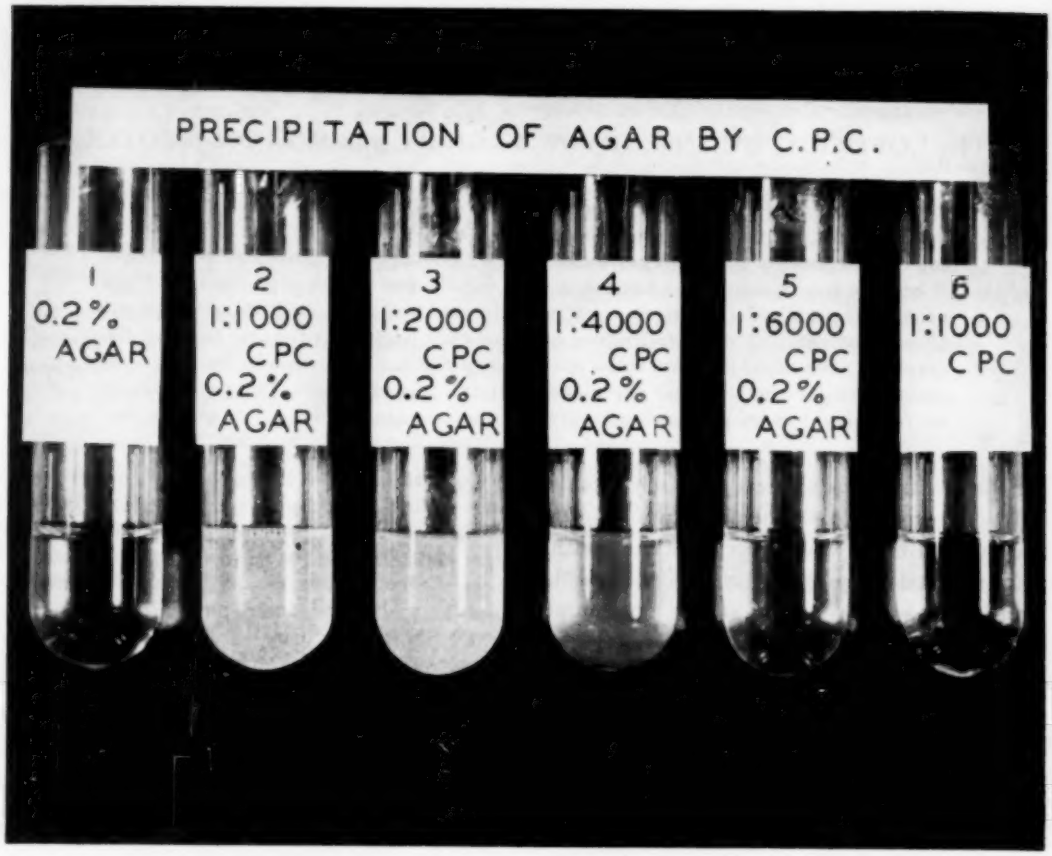

Fig. 1.—C. P. C. —Cetyl pyridinium chloride (Ceepyrn)

spectively, 1:14,000, 1:7000, and 1:6000 in the presence of 0.2, 0.5, and 1.5% agar. At 47° the highest germicidal dilution without agar was 1:95,000. This is not significantly different from the result obtained at 37°. Thus 0.5% agar caused near maximum reduction of germicidal potency.

As undissolved granular agar influenced the antibacterial activity of the cationic germicides to about the same degree as dissolved agar it appeared probable that the surface-active germicides were adsorbed onto the colloidal agar particles and were thus removed from "effective" concentration in the liquid phase.

To test this hypothesis 0.2% granular agar was added to dilutions of Ceepyrn. The mixtures were allowed to stand for thirty minutes and were then filtered. The filtrates and unfiltered mixtures of agar and germicide dilutions were tested for bactericidal activity. Germicidal dilutions of the filtrates were as low as germicidal dilutions of the unfiltered germicide-agar mixtures. In addition the filtrates were analyzed chemically for residual Ceepryn by a modification of the method described by Auerbach (1). These results confirmed the bacteriological findings. The concentrations of Ceepryn in the various filtrates as determined by chemical analysis were much less than the concentrations of Ceepryn in the original solutions. Solutions which originally contained 0.02, 0.01, 0.005, and 0.0033% Ceepryn, after treatment with agar and filtration contained 0.003, 0.003, 0.002, and 0.0016% Ceepryn, respectively.

These data demonstrate that agar actually reduces the concentration of cationic germicide in the aqueous phase and indicate that physical adsorption plays an important role in the reduction of germicidal effectiveness of quaternary ammonium salts.

Addition of various concentrations of Ceepryn to 0.2% agar solutions resulted in the formation of a flocculent granular precipitate. Within the limits tested, higher concentrations of the germicide resulted in greater precipitation of agar as estimated visually (Fig. 1).

It is generally recognized that "skips" or erratic results are obtained when quaternary ammonium salts are tested by the standard procedure. When tested in the presence of blood serum the "skips" or erratic results are decreased to a minimum. It was noted in this experimental work that the presence of agar likewise minimized the erratic results.

DISCUSSION

The observed effects of agar upon quaternary ammonium salts are of considerable theoretical interest and have definite practical importance. The standard test procedure (2) for evaluation of germicidal and antiseptic properties of creams, salves, emollients, etc., calls for the use of an agar cup plate. Obviously an agar plate is inappropriate for the evaluation of products which contain quaternary ammonium salts.

Tobie and Ayres (3) reported a lack of correlation between phenol coefficients and zones of inhibition on agar cup plates for two quaternary ammonium salts. These authors believed unequal diffusion rates to be responsible for the observed discrepancy. Hoogerheide (4) also found that inhibition zones on agar plates failed to correlate with results obtained in broth. This author attributed the lack of correlation to unequal diffusibility of the various compounds. Our data show that either dissolved or undissolved agar reduces the germicidal potency of the quaternary ammonium salts and that this phenomenon occurs when the agar and germicide are completely mixed. Thus it is evident that lack of diffusion or unequal diffusion rates cannot be responsible for erratic results observed with these compounds when tested by agar cup plate methods.

Physical adsorption by agar is suggested as the mechanism responsible for reduction of the bactericidal potency of cationic germicides.

SUMMARY

1. Data have been presented which demonstrate that agar reduces the germicidal potency of quaternary ammonium salts. Experimental results suggest that the reduction is due to physical adsorption.

2. Lack of germicidal activity in the presence of agar may not be attributed to nondiffusibility of these compounds.

3. Agar cup plate tests are inappropriate for evaluation of the germicidal powers of preparations which contain quaternary ammonium salts.

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Book Reviews

The Chemistry of Acetylene, by JULIUS A. NIEULAND and RICHARD R. VOGT. Reinhold Publishing Company, New York, 1945. xi + 216 pp. 15 x 23 cm. Price, $4.00

The chemistry of acetylene is so inextricably tied to the name of Father Nieuland that it would be difficult to know to whom else to turn for an authoritative treatise. It is unfortunate that Father Nieuland never lived to see the completion of this authoritative book.

The book is arranged by a classification of methods of preparation and type reactions of acetylene and is independent of natural historic sequence or industrial position. The main categories in this classification are the physical properties, preparation and purification of acetylene; metallo derivatives of acetylene; the addition of nonmetallic elements and compounds of acetylene; and the polymerization of acetylene and condensation with carbon compounds.

Since acetylene is important in the synthesis of many pharmaceuticals, the first section will be of particular interest to pharmaceutical chemists, while industrialists will be especially interested in the last section which deals with the polymer chemistry which has become the nucleus of synthetic rubber. It was this last phase that Father Nieuland developed so richly and thereby contributed so vastly to the success of the recent war.

The documentation is very complete although contingencies of the times necessarily shortened it to 1938.—MELVIN W. GREEN.

Esquisse De Mes Voyages Au Brésil Et Paraguay, Volume 10, No. 1, by AUGUSTE DE SAINT HILAIRE. The Chronica Botanica Company, Waltham, Mass., G. E. Stechert and Co., New York City, 1946. vi + 62 pp. 17.5 x 26 cm. 6 illus. Price $2.00.

Auguste De Saint Hilaire (1779–1853) takes a special place among the great European naturalists who from the middle of the eighteenth to the middle of the nineteenth century travelled through the still virginal plains of the South American continent observing and describing not only the fauna and flora, the geological formation, and the geographical situation of the areas concerned, but the respective people, their customs and circumstances likewise.

His work was amazing in its volume, and the 25 pages of introduction written by Anna E. Jenkins prove the enormous amount of work done and the results achieved by the explorer as well as his painstaking accuracy. There is, however, still another feature in the writings of Saint Hilaire which makes them especially delightful and which expresses itself in this outline of the explorer's journeys through Brazil and Paraguay in its fullest beauty: a quite unusual charm welling up from a heart as great as it was kind.

This first reprinting of Saint Hilaire's "Introduction" to his "*Histoire des Plantes les plus remarquables du Brésil et due Paraguay*" (1824) will be received by all naturalists having a reading knowledge of French as a literary tidbit.—GEORGE URDANG.

Physical Constants of Hydrocarbons. Volume III, *Mononuclear Aromatic Hydrocarbons*, by GUSTAV EGLOFF. Reinhold Publishing Corporation, New York, 1946. xiii + 661 pp. 15 x 23 cm. Price $15.00.

This volume is the third of a series dealing with the physical constants of hydrocarbons and is limited to mononuclear aromatic compounds. The classification does not contain hydrocarbons having a fused ring structure, any ring of which is aromatic. Polynuclear aromatics, like the indanes, are reserved for Volume IV to be published later.

The data in this volume are from the scientific literature and from the unpublished data of the Universal Oil Products Company and other private research laboratories. The search is said to have involved about 20,000 references, about 5500 of which contained data suitable for publication.

The constants covered by this volume are melting points at all given pressures, boiling points at all given pressures, densities at all given temperatures, refractive indices at several wave lengths of light, rotation angles, critical pressures, critical densities, and sublimation points. All such data are recorded in tabular form and the compounds are named according to the Geneva rules. The evaluation of the accuracy of the data is given in the introduction.

This is a very valuable compilation which belongs in every pharmaceutical research library.—MELVIN W. GREEN.

Textbook of Pharmacognosy, by T. E. WALLIS. J. and A. Churchill, Ltd., London, 1946. vi + 504 pp. 14 x 22 cm. 213 figs. Price, 28*s*.

The author firmly believes that Pharmacognosy is a distinct science but recognizes that it "utilizes the information furnished by the fundamental sciences." He has arranged the crude drugs of vegetable and animal origin according to a system of classification which emphasizes their "structural similarities and differences. Other features such as chemical properties can be included as accessory information."

By classifying the drugs on a morphological basis, he has assembled into groups those items with similar structures such as leaves, flowers, seeds, etc. Other drugs without a definite morphology are grouped according to their method of preparation or according to the nature of their constituents. In the arrangement of these groups the sequence has been from the simpler to the more complex structures.

The series of groups selected and the sequence adopted in this textbook are as follows: Starches; Powders of Natural Occurrence; Fossil Organisms, Shells and Minerals; Hairs and Fibers; Woods; Barks and Galls; Leaves; Flowers; Seeds; Fruits; Entire Organisms; Rhizome and Roots; Unorganized Drugs (Latex, Juices, Extracts); Gums and Saccharine Substances; Resins, Gum-Resins, Oleo-Resins; Fixed Oils, Fats, Waxes; and Glands and Glandular Secretions.

General information concerning each of the above groups included, with few exceptions, the orgin, cultivation and preparation, characters or descriptions, constituents and tests, evaluation, and adulterants. Specific detail devoted to each drug within a group follows the same sequence.

The numerous illustrations, largely line drawings, accompanying the important drugs, are generally of good quality and will surely aid in the laboratory study of the drugs.

In the final chapter devoted to the commerce of drugs, he has clearly and effectively emphasized the advantages of drug plant cultivation. Other factors allied to the commerce of drugs are expertly treated as may be expected from an author closely associated with a trade center such as London.

An appendix which is now devoted only to calcium oxalate could be advantageously enlarged to include other important cell constituents.

The American teacher of pharmacognosy will do well to supplement his information by frequent reference to this textbook.—RALPH F. VOIGHT.